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Cellular Interactions During Motor Nerve Regeneration

Allison F. Rosenberg
University of Pennsylvania, alirosenberg@gmail.com

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Abstract
Vertebrate peripheral nerves can regenerate, enabling severed axons to reconnect with their original synaptic targets. The interactions between injured nerves with cells in their environment, as well as the functional significance of these interactions, have not been determined in vivo and in real time. Here we provide the first minute-by-minute account of cellular interactions between laser transected motor nerves, macrophages, and Schwann cells in live intact zebrafish using transgenic lines that label each cell type in vivo. We find that axon fragmentation triggers macrophage invasion into the nerve to engulf axonal debris, and that delaying nerve fragmentation in a Wlds model does not alter macrophage recruitment but induces a previously unknown `nerve scanning' behavior, suggesting that macrophage recruitment and subsequent nerve invasion are controlled by separate mechanisms, both independent of Schwann cells. A major challenge for regenerating peripheral axons is to identify their original trajectory; Schwann cells are known to provide regenerating axons with factors that stimulate outgrowth, and an adhesive substrate that axons preferentially extend along during regeneration, yet their role in guiding regenerating axons onto their original trajectory is less clear. We show in mutants lacking Schwann cells that axonal growth cones sprout and extend at rates comparable to wild type, but fail to identify their original path, and instead extend along aberrant trajectories. To determine whether Schwann cells function primarily as an adhesive substrate we tested whether a Schwann cell-less axonal scaffold is sufficient to direct axonal growth. These substrates failed to compensate for the absence of Schwann cells, providing evidence that Schwann cells direct regenerating axons towards their original trajectory. To identify signals that guide regenerating motor axons in vivo, we examined mutants lacking the guidance receptor DCC. We find that in DCC mutants a significant fraction of regenerating axons extend along aberrant trajectories. Collectively, this work details the dynamic activities of macrophages and Schwann cells during axon degeneration and early regeneration, while axons are regrowing and selecting their trajectory, and we propose that Schwann cells and DCC mediated guidance are critical early during regeneration, enabling growth cones to navigate towards their original axonal trajectories.

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Allison F. Rosenberg

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Supervisor of Dissertation

_____

Michael Granato, Ph.D., Professor of Cell and Developmental Biology

Graduate Group Chairperson

_____

Daniel Kessler, Ph.D., Associate Professor of Cell and Developmental Biology

Dissertation Committee

Shannon Fisher, M.D., Ph.D., Assistant Professor of Cell and Developmental Biology

Erika Holzbaur, Ph.D., Professor of Physiology

Steven Scherer, M.D., Ph.D., Professor of Neurology, Chief of Neuromuscular Division

Jonathan Raper, Ph.D., Professor of Neuroscience

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Dedication

For all of my teachers, and none more so than my parents and grandparents:

they have taught me how to live.

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ABSTRACT

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Vertebrate peripheral nerves can regenerate, enabling severed axons to reconnect with their original synaptic targets. The interactions between injured nerves with cells in their environment, as well as the functional significance of these interactions, have not been determined *in vivo* and in real time. Here we provide the first minute-by-minute account of cellular interactions between laser transected motor nerves, macrophages, and Schwann cells in live intact zebrafish using transgenic lines that label each cell type *in vivo*. We find that axon fragmentation triggers macrophage invasion into the nerve to engulf axonal debris, and that delaying nerve fragmentation in a Wld^{s} model does not alter macrophage recruitment but induces a previously unknown 'nerve scanning' behavior, suggesting that macrophage recruitment and subsequent nerve invasion are controlled by separate mechanisms, both independent of Schwann cells. A major challenge for regenerating peripheral axons is to identify their original trajectory; Schwann cells are known to provide regenerating axons with factors that stimulate outgrowth, and an adhesive substrate that axons preferentially extend along during regeneration, yet their role in guiding regenerating axons onto their original trajectory is less clear. We show in mutants lacking Schwann cells that axonal growth cones sprout and extend at rates comparable to wild type, but fail to identify their original path, and

instead extend along aberrant trajectories. To determine whether Schwann cells function primarily as an adhesive substrate we tested whether a Schwann cell-less axonal scaffold is sufficient to direct axonal growth. These substrates failed to compensate for the absence of Schwann cells, providing evidence that Schwann cells direct regenerating axons towards their original trajectory. To identify signals that guide regenerating motor axons *in vivo*, we examined mutants lacking the guidance receptor DCC. We find that in DCC mutants a significant fraction of regenerating axons extend along aberrant trajectories. Collectively, this work details the dynamic activities of macrophages and Schwann cells during axon degeneration and early regeneration, while axons are regrowing and selecting their trajectory, and we propose that Schwann cells and DCC mediated guidance are critical early during regeneration, enabling growth cones to navigate towards their original axonal trajectories.

Table of Contents

List of Illustrations

Chapter 2

Chapter 3

Chapter 4

Preface

This thesis regards the process of nerve degeneration and regeneration following a physical injury to the peripheral nervous system. Humans, and other animals, sustain nerve injuries on a regular basis. The most typical traumatic injuries to nerves are crushes and cuts—which happen by way of simple kitchen accidents, car crashes, etc. These injuries can impact our nerves in two different ways: a crush injury squishes a nerve, but leaves the overall structure of the nerve intact, whereas a cut injury severs a nerve, completely separating the cell body from its target synaptic cell, and both injuries leave us incapable of controlling the disconnected target cell, leading to sensory or motor dysfunction. Transection injuries in the peripheral nervous system are the focus on my thesis, and while these are not the most frequent injuries, they are related to other types of nerve injuries, such as traumatic brain injuries that start with focal swellings and eventually lead to axon disconnection. Furthermore, the mechanisms of axon degeneration that are set in motion by a cut or crush injury are shared with many disease-related neuropathies, such as multiple sclerosis, ischemic stroke, glaucoma, and other peripheral motor neuropathies.

Central nervous system injuries are disastrous in mammals, as these nerves do not regenerate, leading to paralysis. However, mammalian peripheral nerves have retained the capacity to regenerate. It's thought that many peripheral nerve injuries may heal without us even knowing that they occurred, and while more serious injuries are capable of healing, surgical interventions are often warranted and peripheral nerves may still not heal very successfully. Transections are also the most difficult nerve injuries to heal

from. Our goal is to understand the mechanisms of nerve degeneration and regeneration resulting from traumatic physical injuries to model not only this process, but how axons are lost in more complex neurodegenerative conditions, and to learn how axons regrow following trauma. To study this process I established a system for studying nerve degeneration and regeneration in the peripheral nervous system of the vertebrate zebrafish, *Danio rerio*.

A nerve is a collection of many long, thin processes called axons, which extend from the cell bodies of the neuron itself and form synapses with, or connections with a target cell, such as a muscle or skin cell. After a transection injury, the axons are severed—part of the axon will remain attached to the neuron's cell body (this is called the proximal end) and part of the axon (the distal end) will be detached from the cell body. The distal half contains the synapse, which is the structure that attaches to and facilitates communication with the target cell. A motor neuron synapses with muscle cells, and a sensory neuron synapses with skin cells. For an injured nerve to heal there are several steps that must occur, and these steps happen both in the nerve's environment around the site of the injury, and also in the injured neuron itself.

Following nerve injury, a process called Wallerian degeneration occurs, encompassing the events that happen to the distal, disconnected side of the axon and the actions of some of the neighboring cells that take part in this process. Once a nerve is cut an inflammatory reaction begins—and leukocytes and macrophages will infiltrate the nerve injury site. The Schwann cell, a glial cell that develops alongside and partners with axons, de-differentiates to a more precursor-like state and upregulates genes that are specific to a regenerative program. The distal half of the axon will fragment into tiny

pieces, the consequence of a cell-autonomous destruction pathway, and these axonal fragments, or blebs, are cleaned up from the environment by macrophages and Schwann cells. As the environment is prepared for the regrowth of the axon, the axon prepares for regrowth as well.

On the proximal side, axons that were severed will retract away from the injury site for a period of time, and eventually, they will re-form a specialized structure called the growth cone that caps the extending tip of the axon. An axon with a growth cone is ready to regrow and extend back into its environment, and this axon, and each axon within the nerve, needs to re-extend through its environment back to its target cell, which is often inches, or even feet away from the neural cell body. Schwann cells are known to help axons regrow back to their targets primarily by providing axons with an adhesive substrate that axons prefer to grow on. One of the major challenges facing axon regeneration is that axons must navigate through their environment back to their target cell, and it must do so with some amount of speed, as tissue that has been denervated for long periods is less receptive to reinnervation. While peripheral axons are capable of regenerating, previous studies have shown that this process is fraught with error. While the processes of axon degeneration and regeneration have been studied for nearly two centuries, we are still unable to clinically intervene in central nervous system injuries, and still lack the knowledge and tools to heal most peripheral nervous system injuries.

Research Summary

This thesis discusses how I established an *in vivo* system in larval zebrafish for studying axon degeneration and regeneration. The zebrafish nervous system is analogous to the human nervous system, with the same cell types present and nerves that grow in similar arrangements as in humans. We focused on motor nerves that innervate the fast muscle fibers in the trunk of the fish, which grow in a segmentally repeating fashion and have stereotyped patterns, and utilized nanoscale lasers to transect these nerves in the live zebrafish. First, we examined the cellular interactions between motor nerves and macrophages following complete nerve transection. We show that as in mammals, myelinated zebrafish peripheral motor nerves undergo Wallerian degeneration, followed by functional regeneration, and that the genetics of motor axon fragmentation is conserved from zebrafish to mammals. We provide the first minute-by-minute account of the destruction speed and synchrony of individual motor axons and Schwann cell morphology changes in a live intact vertebrate animal. Moreover, using overexpression and loss-of function approaches we characterize macrophage recruitment to injured nerves and demonstrate that macrophage recruitment and function occurs independently of Schwann cells. We also uncover a previously uncharacterized plasticity of macrophage behavior as they interact with injured nerves.

Next, we investigated how regenerating axons cross an injury-induced transection gap and select appropriate trajectories. Using zebrafish with genetically ablated Schwann cells and through *in vivo* time lapse movies we find that Schwann cells are critical for guiding axons to appropriate trajectories at the outset of axon regrowth. We show that

trophic support from Schwann cells is dispensable, as axons extend to great lengths in the absence of Schwann cells. By providing an axonal scaffold and genetically ablating Schwann cells we find that a physical adhesive substrate is insufficient to guide regenerating axons, suggesting that Schwann cells also produce signals that guide axons to the appropriate trajectory. Lastly, we examined mutants lacking the netrin receptor DCC and saw that regenerating axons were misguided. Our results indicate that DCC is used as a guidance cue for regenerating axons, and that Schwann cells are indispensable in directing regenerating axons back to their original trajectories *in vivo*.

Chapter 1: Background

What makes axons degenerate?

It is estimated that 20 million people in the US suffer from peripheral neuropathy—in 1995 alone there were over 50,000 peripheral nerve repair surgeries performed (Brannagan, 2012; Evans, 2001). Peripheral nerves can be damaged in a number of ways. Most simply, physical trauma can crush nerves, and more severe injuries can cut through nerves. Nerves can also be damaged by diabetic complications, toxins, which include many chemotherapy drugs, and various diseases. Many diseases fall into the category of a "dying back" disorder, such as some of the Charcot-Marie-Tooth disorders. These are diseases whereby axons first release from the synapse, and eventually the axon degenerates with morphology changes that mirror those of axons degenerating from trauma, such as axonal swelling, microtubule disassembly, and cytoskeletal fragmentation (Cavanagh, 1964; Griffin et al., 1996). In many of these diseases it is unclear whether the disorder is caused by axon degeneration, or whether axon degeneration is a secondary effect of the disease. Interestingly, whether the nerve is damaged by disease, physical trauma, or chemical insult, nerves of both the peripheral and central nervous system undergo the same, stereotyped, evolutionarily conserved and genetically regulated form of self-destruction known as Wallerian degeneration (Coleman, 2005; Waller, 1849). While axons can undergo Wallerian degeneration for a

multitude of reasons, I will focus on transection injuries, which are injuries that fully sever nerves, which we model in our experimental system, though much of what I describe is applicable to crush injuries as well.

Wallerian degeneration

Wallerian degeneration encompasses a number of steps that occur in and around the distal portion of injured axons. While both PNS and CNS axons undergo Wallerian degeneration after injury, Wallerian degeneration progresses much faster in the PNS, and it is thought that this speed is one of the reasons that PNS nerves regenerate successfully, while CNS injuries in mammals result in paralysis (Vargas and Barres, 2007). There are three molecularly distinct phases of axon degeneration: acute axonal degeneration around the injury site, a latent period within the distal axon, and granular disintegration of the distal axon's cytoskeleton.

Within minutes after axotomy the axonal segments proximal and distal to the injury site rapidly degenerate by several hundred micrometers in a process called Acute Axon Degeneration (AAD). AAD lasts between 5-60 minutes after injury, and these blebs of debris are often removed rapidly as well (Kerschensteiner et al., 2005). This rapid disintegration is caused by a channel mediated influx of calcium, which activates the serine-threonine protease calpain, which cleaves axonal neurofilament and microtubule-associated proteins such as spectrin and tubulin (Billger et al., 1988; Johnson et al., 1991; Wang et al., 2012). Following AAD the severed ends of the axons seal off both proximally and distally to the lesion, forming dystrophic bulb structures which

retract away from the lesion site for a period of time, known as the latency or lag time (Griffin et al., 1996). During the latent phase the distal axon remains morphologically intact, axonal transport remains active, and the axon is electrically excitable for brief periods (Smith and Bisby, 1993; Tsao et al., 1994; Tsao et al., 1999). The latency differs in length between organisms: in mouse it lasts about 2 days, whereas in zebrafish larvae it lasts ~2-3 hours (Martin et al., 2010; Rosenberg et al., 2012; Vargas and Barres, 2007). The latency period lasts until the cytoskeleton of the distal, disconnected portion of the axon fragments (Griffin et al., 1996; Lubinska, 1982). In mammalian neural culture microtubule depolymerization is the critical commitment stage in Wallerian degeneration and precedes myelin sheath degeneration and macrophage infiltration; it can occur up to an hour before axon fragmentation (Baloh, 2008; Beirowski et al., 2005; Kerschensteiner et al., 2005; Vargas and Barres, 2007; Zhai et al., 2003). Axons fragment into tiny blebs of axonal debris within milliseconds, and individual axons within the nerve fragment asynchronously, and within 1-2 hours all the axons in the nerve have usually fragmented (Beirowski et al., 2005) (Wang et al.). The length of the latency period is regulatable by temperature, calcium level, and by expression of the Wld^{s} protein (Conforti et al., 2000; George et al., 1995; Tsao et al., 1999). Both calcium ionophores and axon-intrinsic calpains have been shown to cause the breakdown of the axonal cytoskeleton (George et al., 1995; Glass et al., 1993; Perry et al., 1990).

Historically, axon fragmentation was thought to result from nutrient deprivation in the distal axon fragment after disconnection from the cell body (Lunn et al., 1989a; Raff et al., 2002). However, the discovery of a novel, neuronally expressed fusion protein, termed Wallerian degeneration slow (Wld^{s}), which significantly delays Wallerian

degeneration of transected axons, argued against the starvation model and in favor of the idea that Wallerian degeneration is genetically controlled (Lunn et al., 1989a). Research utilizing this protein has shown that axon fragmentation is a genetically regulatable, axon-autonomous process that is distinct from apoptosis (Buckmaster et al., 1995; Deckwerth and Johnson, 1994; Finn et al., 2000; Raff et al., 2002). Wld^S is a chimeric protein, originally identified in C57BL/Ola mice, comprised of the N-terminal 70 amino acids of the polyubiquitination protein Ube4b, a novel 18 amino acid linker from the 5'UTR of Nmnat1 (Nicotinamide mononucleotide adenylyl transferase), and the entirety of Nmnat1, an essential enzyme in the NAD synthetic pathway (Coleman et al., 1998; Conforti et al., 2000; Lunn et al., 1989a). Overexpression of Wld^S in neurons protects axons from degeneration after injury, toxicity, and a number of degenerative diseases, suggesting that these diverse insults activate a common degeneration pathway (Coleman et al., 1998; Coleman and Perry, 2002; Conforti et al., 2000; Glass et al., 1993; Lunn et al., 1989a; Perry et al., 1990; Raff et al., 2002; Vargas and Barres, 2007). Wld^S does not delay developmental pruning, distinguishing between the two processes (Hoopfer et al., 2006a). Axon fragmentation following the latency period is seen in neuron-only culture, and the Wld^S protein is only protective when expressed in axons themselves, indicating that fragmentation is regulated autonomously (Glass et al., 1993; Hoopfer et al., 2006a; Perry et al., 1990);(Buckmaster et al., 1995). Wld^S delays fragmentation in multiple model systems, indicating that the axon destruction program is evolutionarily conserved (Adalbert et al., 2005b; Araki et al., 2004a; Hoopfer et al., 2006a; Lunn et al., 1989a; MacDonald et al., 2006b; Martin et al., 2010; Raff et al., 2002; Wang et al., 2001a). Additionally, Wld^S is only axon protective, it has no effect on the cell body itself

(Adalbert et al., 2005b; Deckwerth and Johnson, 1994). How Wld^s delays degeneration and what organelles and proteins it interacts with are still being examined, and was recently reviewed (Coleman and Freeman, 2010). Currently, determining whether expression of the Wld^s protein in a given circumstance is axono-protective is a useful tool for determining whether the process being studied utilizes the same degenerative mechanisms as other injury types. We utilized this protein to determine that zebrafish nerves degenerate using the same genetic mechanism as other vertebrates (Rosenberg et al., 2012).

What ultimately triggers axon fragmentation remains unknown. Two theories predominate: either a pro-degeneration signal is generated at the lesion site, and travels through the distal axon until it builds up at a critical level to trigger fragmentation, or, a cell body-derived survival factor is depleted during the latency period, and when levels of the survival factor drop below threshold axon fragmentation is triggered (Coleman and Freeman, 2010). While there is evidence to support both theories neither has been fully accepted by the community. However, a few pieces of evidence argue in favor of the loss of a survival factor: axon degeneration can be triggered without physical injury, axon transport disorders can cause degeneration, and protein synthesis impairment also triggers degeneration. Additionally, colder temperatures and proteasome inhibition extends the latent phase, both of which could extend the lifetime of a survival factor (Coleman and Freeman, 2010; Lubinska, 1982). Following the yet-unknown trigger event, a large calcium spike travels through the distal axon. This calcium is thought enter the axon through channel mediated calcium influx, as well as intracellular releases of calcium

stores within the distal axon. This directly precedes the fragmentation of the axons into blebs.

The role of macrophages and Schwann cells in Wallerian degeneration

Axon fragmentation leaves behind cellular and membraneous debris. In the CNS, this debris is inhibitory to regrowing axons. PNS myelin debris contains molecules like MAG that are traditionally inhibitory to outgrowth, as seen *in vitro* (Bähr and Przyrembel, 1995; David et al., 1995; Shen et al., 1998), and axon growth into intact Wld^{s} peripheral nerves in certain cases is limited, possibly due to the still-intact myelin that coats these nerves (Bisby and Chen, 1990; Brown et al., 1991; Chen and Bisby, 1993a). However, it remains unclear whether this debris is inhibitory *in vivo* (Kang and Lichtman, 2013). In the PNS, this debris is rapidly cleared by Schwann cells and macrophages: in mammals it is cleared in ~7-14 days, whereas in the CNS clearance takes months to years (Beuche and Friede, 1984; Beuche et al., 1986; George and Griffin, 1994a; Griffin et al., 1992; Lubinska, 1977; Waller, 1849). The speed of debris clearance is one of many critical differences between Wallerian degeneration in the CNS and the PNS, and this rapid debris clearance in the PNS is thought to generate an extracellular milieu conducive for axonal regeneration (Hirata et al., 1999; Holtzman and Novikoff, 1965; O'Daly and Imaeda, 1967; Stoll et al., 1989a; Stoll et al., 1989b).

Schwann cells are neural crest derived glial cells that partner with axons for the life of the organism. During development Schwann cell precursors migrate alongside axons, and as they differentiate Schwann cells sort axons by size, or caliber. Terminally

differentiated Schwann cells ensheath axons in myelin, a fatty membrane that allows for rapid conduction of axonal action potentials. Schwann cell-provided myelin and trophic support are essential for proper axon function: many neuropathies, such as the CMT disorders, are caused by Schwann cell dysfunction and lead to axonal problems (Nave and Trapp, 2008). In the PNS, it's thought that axon-derived NeuregulinI typeIII regulates Schwann cell survival during development, however, Schwann cell maturation is accompanied by autocrine survival circuits that involve PDGF, IGF-1, and NT3, such that Schwann cells survive to play active roles in regeneration (Jessen and Mirsky, 1999). However, following periods of chronic denervation lasting several months or more, increased Schwann cell death is ultimately observed (Ebenezer et al., 2007). Following axonal injury Schwann cells de-differentiate and stop producing myelin, and also degrade the myelin surrounding injured axons (Martini et al., 2008; Scherer et al., 1984; Stoll et al., 1989a; Trapp et al., 1988). Schwann cells degrade myelin and axon debris with hydrolytic enzymes in intracellular vacuoles, and pump debris into the extracellular space for macrophages to phagocytose (Holtzman and Novikoff, 1965; O'Daly and Imaeda, 1967). Interestingly, delaying axon degeneration prevents the progression of these and other glial events, suggesting that axon fragmentation is required to trigger Schwann cell activation during Wallerian degeneration (George and Griffin, 1994b; Lunn et al., 1989a). How Schwann cells recognize axonal injury remains unclear. It is possible that changes in axonal NeuregulinI typeIII, which can bind to Schwann cell ErbB2/B3 receptors may transmit this signal, but it is unclear whether this is the definitive injury signal (Guertin et al., 2005; Vargas and Barres, 2007).

Macrophages have been reported to accumulate at injured nerve after fragmentation, and their recruitment is thought to depend on signals released from Schwann cells, though nerves themselves also produce chemotactic molecules (Avellino et al., 1995; Banner and Patterson, 1994; Hirata and Kawabuchi, 2002; Lunn et al., 1989a; Monaco et al., 1992; Perry et al., 1987; Shamash et al., 2002; Stoll et al., 1989a; Stoll et al., 1989b; Subang et al., 1999; Tofaris et al., 2002). Macrophages are recruited in mammals to degenerating sciatic nerves by 3 days post axotomy, after axon fragmentation, and their numbers peak 14-21 days later (Avellino et al., 1995; Monaco et al., 1992; Perry et al., 1987; Stoll et al., 1989a; Stoll et al., 1989b). In partial injuries macrophages are associated with injured fibers, indicating that local cues guide them to damaged axons, and not uninjured fibers (Stoll et al., 1989b). At the lesioned nerve macrophages phagocytose axonal debris and myelin ovoids. Macrophages express cell surface receptors for opsins, which are proteins that coat the extracellular surface of debris, labeling it for phagocytosis by macrophages (Bruck and Friede, 1990; Friede and Bruck, 1993). The complement system is also involved in the inflammatory response to axonal injury and opsonization, as are IgM antibodies that appear on the surface of debris (Bruck, 1997; Smith et al., 1998; Vargas and Barres, 2007). In our system we have examined the recruitment of macrophages after injury and witnessed their arrival at injured nerves within minutes after transection, long before they have been seen in other studies. We also verified the specificity of macrophage recruitment to lesioned fibers, and determined that distinct signals likely mediate macrophage recruitment to the injury site and macrophage infiltration into the fragmenting distal nerve.

Peripheral neuron survival after injury

Neurotrophins are substances that are necessary for the survival or maintenance of neurons. During development neuronal survival is regulated by target-derived neurotrophic support, but nerves become less dependent on this support in adult animals. Axotomy not only separates neural cell bodies from target-derived trophic signals, but also induces injury signals. In young rats (prenatal through first few weeks of life) axotomy causes the death of at least 30% of neurons, whereas in adult rats (over 3 weeks of age) neurons survived axotomy, but began to die after about a month of being prevented from regeneration (Koliatsos et al., 1993; Snider and Thanedar, 1989). Some reports indicate that the distance of the injury from the cell body impacts cell survival, which is not seen in other studies (Tornqvist and Aldskogius, 1994). It is likely that differing results arise from studies utilizing multiple animal species of various ages, and from different types of injuries on different nerves at various distances from the cell bodies, and lastly, differing methods for counting cell bodies. In consensus, axotomy in neonatal and very young animals results a great proportion of neural death, whereas axotomy in adult animals results in overall neural survival, unless axons are prevented from regenerating for more than 2-3 weeks. It also seems accepted that distal injuries result in greater neural survival compared to injuries more proximal to the cell body (Lowrie and Vrbova, 1992). It is unclear whether injured neurons die due to a lack of a connection to or reception of some factor from the target cell, or whether death is an eventual reaction to the injury itself, if not healed by reinnervation (Kuno, 1990).

Schwann cells and macrophages are thought to provide trophic support to injured axons (Arthur-Farraj et al., 2012; Brown et al., 1991; Jessen and Mirsky, 2008). Tyrosine kinase receptors in the axonal membrane bind trophic factors that can inhibit apoptosis machinery, such as the pI3K/AKT pathway and the ras/raf/MAPK pathway (Goldberg and Barres, 2000). Additionally, PNS neurons are believed to establish autocrine circuits that promote their own survival after injury: the axonal stump produces survival factors, such as the neurotrophin BDNF, and retrogradely transports these factors to support survival (Acheson et al., 1995; Kuno, 1990). We find that very few motor neurons die after injury in our system, suggesting that these motor neurons are fairly mature.

Axon-intrinsic signaling that promotes regeneration

Following injury calcium flows into the injured axon, and stops when axons reseal (Bradke et al., 2012). In calcium-free culture conditions axotomized mammalian neurons do not generate new growth cones (Rossi et al., 2007). High intracellular calcium has been shown to activate PKA and ERKs, which regulate cytoskeletal modifications and axon protein synthesis (Chierzi et al., 2005). Transcripts for various cytoskeletal elements, signal transduction, anti-oxidant enzymes, and neurodegenerative disorder transcripts have been identified in axons (Hammarlund et al., 2009; Rossi et al., 2007). Many of these transcripts are regulated by external stimuli like guidance cues, neurotrophins, and axotomy, suggesting that axon injury activates a labile, local axonal regenerative response (Rossi et al., 2007). These locally translated mRNAs and proteins may have a second role in relaying injury signaling to the cell body. Locally translated

mRNAs and proteins can be shuttled by importin alpha and the minus-end directed motor protein dynein in macromolecular complexes to the cell body, where they have been shown to interact with mitogen activated protein kinases (MAPKs), amongst other proteins. One of these complexes, containing a motor protein, Sunday driver (syd), and JNK (cJun N-terminal kinases), ultimately phosphorylates c-Jun in the nucleus, an event that is important for regenerative signaling. Thus, these complexes may convey axon injury signals to the cell body (Rossi et al., 2007). The reaction of the cell body to axotomy, or chromatolysis, can be blocked by application of actinomycin D, suggesting that newly synthesized RNAs and proteins are responsible for the response to injury (Kuno, 1990).

Axonal regrowth requires restructuring of the actin and microtubule cytoskeleton, as well as new membrane. Regenerating axons first make transient filopodia, which then turn into growth cones. In mammalian sensory axons the kinases MAPK1/ERK2, MAPK3/ERK1, MAPK11–14/ p38 MAPKs and mTOR are required for growth cone formation (Bradke et al., 2012). In the proximal axon the MAPKKK Dual leucine kinase (DLK) was shown to function specifically in regeneration, and not development, to cell-autonomously initiate growth cones in *C. elegans* after axotomy. Without DLK axons make short, transient filopodia that fail to persist and form growth cones. Similar results were seen in axons lacking p38 MAPK (Hammarlund et al., 2009). DLK can activate cJun N-terminal kinases (JNK) and p38 MAPK (Fan et al., 1996; Shin et al., 2012). In Drosophila, axonal injury upregulated levels of the DLK homolog Wallenda, which activates a retrograde signaling pathway, transports p-JNK to the nucleus, and leads to a transcriptional response (Xiong et al., 2010). In mouse, DLK was shown after injury to

promote the retrograde transport of p-STAT3 and a scaffolding protein JIP3that links DLK and JNK to axon transport machinery, activating yet another pro-regenerative signaling pathway (Shin et al., 2012). In theory, axotomy blocks trafficking of DLK at the stump, where DLK homodimerizes and activates downstream signals, such as p38 MAPK, which acts both in the axon as a regulator of local protein synthesis, as well as retrogradely transporting p-JNK and P-STAT3 back to the nucleus, where these proteins contribute to injury-induced changes in gene transcription. P38 MAPK regulates microtubule dynamics, a requirement for growth cone initiation (Hammarlund et al., 2009). Other signaling pathways shown to promote axon regrowth include neutrotrophins, cAMP, and Akt (Bhatt et al., 2004; Rossi et al., 2007; Tucker and Mearow, 2008).

Regrowth also requires new membrane, which is provided by golgi-derived vesicles (Bradke et al., 2012). Interestingly, following injury glial cells upregulate apolipoprotein E (ApoE) and secrete lipoproteins, cholesterol, and phospholipids. Axons can utilize these supplies to form new plasma membranes and increase their own regeneration and growth rates (Vance et al., 2006). Apolipoprotein D mRNA has also been seen in endoneurial fibroblasts after regeneration (Spreyer et al., 1990). ApoE and ApoA-I are upregulated in rat Schwann cells and macrophages around sciatic nerve lesions, and axons expressed LDL receptors, indicating that the machinery for lipid transfer is present (Boyles et al., 1989). The necessity of lipid re-uptake is unclear, as regeneration appears normal in ApoE, ApoAI, and LDLR knockout mice, suggesting that there may be redundancy in lipid recycling mechanisms or that other apolipoproteins are involved (Goodrum et al., 1995; Goodrum et al., 2000a; Goodrum et al., 2000b).

The role of macrophages, Schwann cells, and other cell types in axon regeneration

While neural survival and growth cone reformation are largely cell-autonomous processes, various cell types collaborate in axon regeneration. Many non-axonal cells, such as macrophages (Beuche and Friede, 1984; Perry and Brown, 1992b; Perry et al., 1987; Stoll et al., 1989a; Stoll et al., 1989b), Schwann cells (Brosius Lutz and Barres, 2014), fibroblasts (Abernethy et al., 1994; Martini et al., 1990; Parrinello et al., 2010) and perineural ensheathing glia (Schröder et al., 1993; Weis et al., 1994b) have been shown to play roles in regeneration.

Recruitment of macrophages has been linked to successful nerve regeneration (Perry and Brown, 1992b). Wld^{s} overexpression in sensory axons reduces macrophage recruitment and also delays functional regeneration, suggesting that the processes of axon degeneration, macrophage recruitment, and regeneration are intricately interconnected (Bisby and Chen, 1990; Brown et al., 1992; Chen and Bisby, 1993a, b). Macrophages are recruited from the circulation into the degenerating sciatic nerve typically a day after axon fragmentation, and their numbers peak 14-21 days after axotomy (Avellino et al., 1995; Monaco et al., 1992; Perry et al., 1987; Stoll et al., 1989b). Macrophages are recruited first to sites of injury, then to the distal nerve after distal axon degeneration, mirroring the progression of Wallerian degeneration (Perry and Brown, 1992a). An injury to a peripheral axon, followed a week later by an injury to a central axonal branch of the same cell, called a conditional or priming lesion, leads to enhanced regeneration from the usually recalcitrant central axon. Interestingly, this enhanced regrowth from the priming

lesion can be mimicked by injection of inflammogens or macrophages in the intact ganglion (Lu and Richardson, 1991). This data, and the fact that macrophages remain at injured nerves after debris is removed, suggest that macrophages play additional roles in stimulating growth from injured axons. We investigate macrophage recruitment and demonstrate that macrophages arrive at nerves within minutes after injury, and that recruitment is independent of Schwann cell signaling. We also describe a new behavior of macrophages at Wld^s nerves that allows us to distinguish between initial macrophage recruitment to the injury site and macrophage invasion of the distal fragmenting nerve.

Macrophages produce interleukin-1, which has been shown to *in vivo* to trigger NGF production by Schwann cells (Lindholm et al., 1987). In C57BL/Ola mice, the lack of macrophage recruitment results in low levels of interleukin-1and NGF mRNA in the distal nerve stump of a cut nerve *in vivo* (Brown et al., 1991; Heumann et al., 1987). Macrophages that have digested myelin produce a soluble factor that acts as a Schwann cell mitogen, as well as TGF-B, which has been shown *in vitro* to stimulate Schwann cell mitosis, suggesting that macrophages play a role in stimulating the increase in Schwann cell numbers (Perry and Brown, 1992a). Additionally, the cellular influx of myeloid cells has been accompanied by peaks in the expression of interleukins 1β, 6 and 10, IFγ, TNF-α, RAGE, LIF, MCP-1, MIP1α, MMPs, and NO, to name a few: all molecules that influence repair and regeneration (Zochodne, 2012).

As previously discussed, Schwann cells assist an influx of inflammatory cells, such as macrophages, to phacogytose and degrade axon and myelin debris, thus preparing the extracellular milleu for the arrival of regenerating axons (Hirata and Kawabuchi, 2002; Hirata et al., 1999; Holtzman and Novikoff, 1965; O'Daly and Imaeda, 1967; Stoll

et al., 1989b). However, these Schwann cells do more than coordinate the debris cleanup effort. Early studies established that the environment generated by macrophages and Schwann cells is critical for successful nerve regeneration (Aguayo et al., 1981; David and Aguayo, 1981). Following nerve injury Schwann cells dedifferentiate to a more precursor-like state and upregulate genes coordinated by the transcription factor c-jun that are specific to a regenerative role (Arthur-Farraj et al., 2012; Brosius Lutz and Barres, 2014). While remaining along the nerve's original path, the distal Schwann cells, de-differentiated and deneverated, form an adhesive substrate for axonal regrowth known as the band of Bungner (Cajal, 1928). These Schwann cells do not resemble Schwann cells during development, and are postulated to be a transdifferentiated form of the Schwann cell, referred to as the repair or Bungner cell, with their own morphology and expression patterns (Arthur-Farraj et al., 2012). Schwann cells do not seem to be important for neurite extension, per se, but regenerating axons grow in close association with the band of Bungner along the inner surface of the basal laminar tubes (Bresjanac and Sketelj, 1989; Scherer et al., 1984). In this way, axons retrace their former routes and can find and reinnervate appropriate target cells (Nguyen et al., 2002).

The Bungner form of the Schwann cell produces molecules directly implicated in repair such as: surface proteins N-cadherin, p75NTR, and NCAM, signaling molecules and neurotrophins such as GDNF, artemin, sonic hedgehog, and BDNF, and adhesive molecules that promote axon growth, like laminin and ninjurin (Vargas and Barres, 2007). Experiments that kill Schwann cells, but leave their basal lamina intact, showed that the basal lamina is sufficient to enhance nerve regrowth, and that the adhesion of axons to a substrate is critical for axon outgrowth. Axon outgrowth is also enhanced on

nerve grafts that lack Schwann cells, but are seeded with laminin (Hall, 1986b; Ide et al., 1983; Scherer et al., 1984). These studies suggest that ECM produced by Schwann cells is important for axon outgrowth. Both Schwann cells and axons express integrins, which are receptors for laminin and fibronectins, which are critical for adhesive interactions with the basement membrane (Wildering et al., 2002). Laminin and fibronectins have been shown to be greatly expressed in nerve outgrowth zones after nerve injury, especially compared to intact nerves (Liu et al., 2009). Axons preferentially elongate, and elongate faster, on strongly adhesive basal laminar substrates (Letourneau, 1975; Scherer et al., 1984). While it seems as though axonal adhesion to the ECM produced by Schwann cells is the most critical aspect of their regrowth, axons preferred to grow over grafts with Schwann cells present over acellular ECM grafts, suggesting that Schwann cells produce more than an adhesive substrate to promote axon regeneration (Bresjanac and Sketelj, 1989; Brown and Hopkins, 1981; Dahlin et al., 1995; Ide et al., 1990; Kerns et al., 1993; Sketelj et al., 1989). Sensory axons don't regenerate well in Wld^{s}, (Brown et al., 1991), but when they did regenerate, it seems as though axons grew over the Schwann cells (Fruttiger et al., 1995). In sum, these studies showed that pre-degenerated peripheral nerve tissue with Schwann cells were highly conducive to axonal regrowth, indicating that these distal Schwann cells, activated by injury, were doing something to enhance regeneration. We explore this topic as well, and determine that Schwann cells indeed provide more than a simple adhesive substrate for regrowing axons, and also provide direction to regrowing sprouts early, before sprouts approach the band of Bungner.

Following a crush injury, the basal lamina surrounding Schwann cells and degenerating axons remains intact, and axons regrow along the band of Bungner towards their former synaptic partners with success (Cajal, 1928; Nguyen et al., 2002; Scherer et al., 1984). However, following a more severe transection injury, a gap is formed between the proximal nerve stump and the band of Bunger, not only by the injury itself but also due to axon retraction on either side of the transection site (Cajal, 1928). The success of axonal pathfinding across this gap to the band of Bungner is critical to the success of regeneration. A long standing question has been how axons cross this gap: whether alone, in tandem with Schwann cells, or whether Schwann cells lead the way. Most studies have found that axons and Schwann cells usually emerge from the proximal stump and grow outward together (Chen et al., 2005; Hall, 1986a; Son et al., 1996; Torigoe et al., 1996; Webber et al., 2011).

For Schwann cells to lead regenerating axons, Schwann cells must be able to either extend their membranes great distances or migrate. De-differentiated Schwann cells re-enter the cell cycle and proliferate (Oaklander and Spencer, 1988). Previous studies have shown that blocking Schwann cell proliferation interrupts their ability to migrate and support axon regeneration (Hall, 1986a; Ide et al., 1983; Zochodne, 2012). In these studies nerves were irradiated or a cellular proliferation inhibitor was pumped into outgrowth chambers containing injured nerves. In these chambers Schwann cells neither proliferated, nor migrated, and axon outgrowth was also disrupted (Chen et al., 2005; Hall, 1986a). While we cannot rule out that these treatments had effects on the axons themselves, on the surrounding tissue, or Schwann cell migration itself, these experiments suggest that Schwann cell proliferation, migration, and axon regeneration are

linked. These studies prompted us to investigate the role of Schwann cells in axon regeneration: it seemed clear that Schwann cells are capable of more than debris clearance, but their role remained unclear. Our system allows for the genetic removal of Schwann cells *in vivo*, and by examining axon regeneration in their absence we can resolve how these cells interact with axons and support axon regeneration.

There are a few other cells that have been shown to have a role in axon regeneration. In addition to Schwann cells, fibroblasts help axons extend from the proximal stump and cross the transection gap (Cajal, 1928). Fibroblasts form caps on the proximal and distal nerve stumps, and along with Schwann cells form bridges between the stumps in an ephrin-B/EphB dependent process (Son et al., 1996); (Abernethy et al., 1994; Martini et al., 1990; Parrinello et al., 2010). Perineural ensheathing glia are CNS derived glial cells that extend cytoplasmic protusions to the periphery to ensheath both the motor nerve and Schwann cells in a protective blood-nerve barrier (Akert et al., 1976; Kucenas et al., 2008). To date, perineural glia have not been tested for roles in Wallerian degeneration, but after sciatic nerve transection *in vitro* perineural glia have been shown to form cellular bridges that guide axon regeneration (Schröder et al., 1993; Weis et al., 1994a). It remains unclear whether perineural glia play a role in axon regeneration *in vivo*. Perineurial mini-fascicles have been observed in both the proximal and distal portions of the regenerating nerve, and perineural glia have been show to cross a transection gap, but whether these cells are leaders or followers remains unclear (Binari et al., 2013 ; Geuna et al., 2009). It's also been shown that injured tissue itself plays a role in regeneration. Wounded tissue produces H_2O_2, which has been shown to promote axon growth and mediate leukocyte recruitment (Rieger and Sagasti, 2011).

Molecular cues that direct regrowing axons

Following nerve injury disconnected axons must regrow through their environment back to their synaptic partner cells. While axons rely in large part on Schwann cells for regeneration, after transection Schwann cell and basal lamina continuity is lost, and retraction of proximal and distal nerve stumps from the transection site forms a gap. The upper limit for successful growth across nerve gaps in rats generally is 10–15mm (Zochodne, 2008). Axons can cross shorter gaps and reach the distal stump, but may enter incorrect Schwann tubes and innervate the wrong targets (Brushart, 1988, 1993). Thus, selection of the appropriate trajectory that will lead the axon to its synaptic partner cell presents a significant challenge.

During development axons pathfind using cocktail of instructive and inhibitory cues embedded in their environment, and guidance receptors on axonal growth cones interpret these cues to maintain axons along proscribed pathways (Tessier-Lavigne and Goodman, 1996). It remains unclear what guidance cues are available to regenerating axons, and what cues regenerating axons remain sensitive to. Many developmental guidance cues and associated receptors are shown to be altered after axonal injury. Peripheral axons will come in contact with netrin1, neuropilins 1 and 2, NCAM, L1, MAG, and CSPGs, but how these guidance molecules affect axons in the changed regenerative environment remains to be elucidated (Brushart, 2011). For example, during development *C. elegans* ALM mechanosensory axons are repelled by slits and attracted by DCC/netrin signaling, but these axons do not re-use DCC and netrin for regeneration

(Gabel et al., 2008). Slit2 was found to be upregulated by Schwann cells after transection but not after a crush injury, and to stimulate sprouting; Netrin1 was also upregulated after transection, but not crush, in distal Schwann cells (Madison et al., 2000; Park et al., 2007). In explants of adult DRG neurons that express the Unc5 receptor netrin treatment inhibits axon outgrowth, and *in vitro*, spinal motor neurons extend axons further when netrin levels are decreased, indicating that netrin signaling may be inhibitory to some adult neurons through the Unc5 receptor (Shim and Ming, 2010). In mouse DCC/Netrin signaling provides specificity to the direction of axon regrowth, while Netrin/Unc5H signaling is inhibitory and repellent, indicating that these mechanisms are re-used in development and the relative levels of each netrin receptor is critical to the axon's perception of the netrin ligand (Webber et al., 2011). These results suggest differences between the requirements of different cell types between development and regeneration, and could potentially provide specificity within a mixed nerve population. How guidance cues are re-used during regeneration *in vivo* remains murky. We investigate the netrin receptor DCC in axon regeneration and find that DCC is required for guiding regrowing motor axons.

Synapse reformation and remyelination

While synapse reformation and remyelination are outside of the purview of this thesis, these processes are equally essential to successful axon regeneration. Following axonal regrowth through the band of Bungner axons and Schwann cells appear to follow similar mechanisms as seen during development: Schwann cells re-differentiate, sort and

ensheath axons in myelin, though post-regeneration myelin internodes tend to be shorter than those formed in development. Neuregulin/ErbB signaling is thought to promote Schwann cell remyelination. Axons are capable of reinnervating appropriate targets through two mechanisms: axons can regrow to and synapse with their appropriate target cells, or axons grow to and reinnervate multiple locations, and later appropriate synapses outcompete inappropriate synapses, which withdraw (Salzer, 1999). A separate mechanism, emblematic of the plasticity of the nervous system, can also restore target cell function: nearby uninjured axons can produce collateral sprouts that synapse with denervated, ectopic locations (Zochodne, 2012).

Clinical outcomes, and why more research is needed

Despite the universally touted fact that "PNS neurons can regenerate, CNS neurons do not," capacity alone does not lead to successful regeneration. While motor and sensory axons of the PNS have been shown to reconnect appropriately with muscle cells or sensory cells (Höke et al., 2006), very often there are mix-ups between motor pools and target muscle cells, leading to dysfunction, or mix-ups between sensory and motor pools leading to synkinesis (Rossi et al., 2007). Axonal misrouting, increased branching, changes or deficiencies in guidance systems, and the failure to refine synapses can all lead to less successful outcomes. Interestingly, electrical stimulation has been shown to enhance reinnervation specificity, as does forced use of the muscle with specific training regiments, which have been shown to promote axon regeneration and the

reorganization of cortical circuits that control behavior, increasing plasticity within the system (Rossi et al., 2007).

Clinically there are more barriers to regeneration than in the lab. Human injuries are often more complex, often including crush and stretch injuries in tandem, or other combinations of problems. Peripheral nerve lesions occur in ~3% of multiple trauma patients (not including patients with spinal cord injury, and patients not associated with multiple trauma) (Noble et al., 1998). Though thousands of surgeries are performed each year in American emergency rooms, it's estimated that only 10% of axons reach target organs after surgery (Witzel et al., 2005). Although speed of axon extension is often looked to as the limiting factor in PNS regeneration, there may be more factors in play, including damage to the target tissue and long term denervation, which can make target tissues inhospitable to regenerating axons. Non-traumatic neuropathies make up the majority of the population with peripheral neuropathy: this population includes polyneuropathies, focal entrapment, and ischemic neuropathies. Over 400 million people have diabetic polyneuropathy globally (Zochodne, 2012). In sum, we have much more to learn.

Zebrafish as a model system for studying axon degeneration and regeneration

Wallerian degeneration and subsequent axon regeneration are complex, multistep processes guided by yet to be defined genetic pathways. While Wallerian degeneration has been extensively studied in model systems such as the rat, mouse, *C. elegans* and Drosophila, there are many open questions, in part due to the limited ability to

continuously visualize degeneration and regeneration in live animals, in real time. Zebrafish embryos and larvae are optically transparent, by four days post fertilization they have already acquired simple sensory and locomotor capabilities, and between five days and two weeks post fertilization they exhibit behaviors like hunting for food and escaping predators, much like their adult counterparts (Guo, 2004). In lower vertebrates, like zebrafish, the CNS can regenerate as well as the PNS, and for this reason I focused on PNS motor neurons, which accurately model human motor neurons. As zebrafish are facile organisms for genetic manipulation, many transgenic lines that label neural, glial, and inflammatory cells exist, as well as mutants lines that ablate these populations or modulate aspects of cell function (Jing et al., 2009; Lefebvre et al., 2007; Schneider and Granato, 2006). To establish zebrafish as a model for Wallerian degeneration in the PNS I use 5 day post fertilization (dpf) zebrafish larvae, which are transparent, have fully extended motor axons, and have functional neuromuscular junctions (Liu and Westerfield, 1987; Liu and Westerfield, 1990). To visualize individual motor axons I inject a dsRed construct targeted to motor neurons under the Mnx1 promoter (Mnx1:dsRed) at the one cell stage into transgenic embryos that express GFP in all motor neurons (Tg(Mnx1:GFP)) (Flanagan-Steet et al., 2005). This creates larvae in which all motor nerves are labeled in green and individual motor neurons are labeled in red, an approach that allows for analysis of the entire motor nerve in green and single axon dynamics in red, increasing overall resolution. When larvae are five days old I anaesthetize them, immobilize them in agarose, and use a laser to completely transect selected motor nerves in the trunk of the larvae while limiting damage to surrounding tissues. I image the injured nerves with a spinning disc confocal microscope for several

hours with a 60X lens to obtain a detailed description of axon degeneration. After 6-8 hours of imaging the larvae are freed from the agar, returned to a petri dish, and fed. The locations of the lesioned axons in each larva are recorded, and the same nerves can be repeatedly imaged over subsequent days and the larvae raised to adulthood. Schwann cells, perineural glia, and macrophages are also present, as is myelin, thus the five day old PNS has the characteristics of a mature, adult PNS (Antonellis et al., 2008; Kucenas et al., 2008; Pogoda et al., 2006).

Chapter 2:

In vivo nerve-macrophage interactions following peripheral nerve injury

The data in Chapter Two have been published in the *Journal of Neuroscience*.

Allison Rosenberg, Marc Wolman, Clara Franzini-Armstrong, and Michael Granato

Abstract

In vertebrates, the peripheral nervous system has retained its regenerative capacity, enabling severed axons to reconnect with their original synaptic targets. While it is well documented that a favorable environment is critical for nerve regeneration, the complex cellular interactions between injured nerves with cells in their environment, as well as the functional significance of these interactions, have not been determined *in vivo* and in real time. Here we provide the first minute-by-minute account of cellular interactions between laser transected motor nerves and macrophages in live intact zebrafish. We show that macrophages arrive at the lesion site long before axon fragmentation, much earlier than previously thought. Moreover, we find that axon fragmentation triggers macrophage invasion into the nerve to engulf axonal debris, and that delaying nerve fragmentation in a Wld^{s} model does not alter macrophage recruitment but induces a previously unknown 'nerve scanning' behavior, suggesting that macrophage recruitment and subsequent nerve invasion are controlled by separate mechanisms. Finally, we demonstrate that macrophage recruitment, thought to be

dependent on Schwann cell derived signals, occurs independently of Schwann cells. Thus, live cell imaging defines novel cellular and functional interactions between injured nerves and immune cells.

Introduction

In response to injury, axons of both the central nervous system (CNS) and the peripheral nervous systems (PNS) undergo a stereotyped and genetically regulated form of self-destruction known as Wallerian degeneration (Waller, 1849). Key to this process is the Wld^S protein, originally identified in C57BL/Ola mice, which delays axonal fragmentation through a cell autonomous mechanism (Coleman et al., 1998; Conforti et al., 2000; Lunn et al., 1989a; Perry et al., 1990; Raff et al., 2002). While the axon autonomous mechanisms of this self-destruction program are been studied extensively, the cellular and molecular interactions between injured axons and non-neuronal cells such as glial and immune cells are less well characterized. In the periphery, Schwann cells and macrophages remove the cellular and membranous debris of the fragmented nerve, thereby generating an extracellular milieu conducive for axonal regeneration (Hirata and Kawabuchi, 2002; Hirata et al., 1999; Holtzman and Novikoff, 1965; reviewed: O'Daly and Imaeda, 1967; Stoll et al., 1989a; Vargas and Barres, 2007).

Besides their phagocytic role, Schwann cells are thought to recruit immune cells to the lesion site (Banner and Patterson, 1994; Shamash et al., 2002; Subang et al., 1999; Tofaris et al., 2002), and macrophage infiltration and rapid debris removal are considered key prerequisites for nerve regeneration (reviewed: Perry and Brown, 1992a). For

example, Wld^s overexpression in sensory axons reduces macrophage recruitment and delays functional regeneration, suggesting that the processes of axonal degeneration and regeneration are intricately interconnected (Bisby and Chen, 1990; Brown et al., 1992; Chen and Bisby, 1993a, b). Yet despite their importance for nerve degeneration and regeneration, the cellular interactions between injured nerves and macrophages have not been determined *in vivo* and in real time.

Here, we examine the cellular interactions between motor nerves and macrophages following complete nerve transection. We show that as in mammals, myelinated zebrafish peripheral motor nerves undergo Wallerian degeneration, followed by functional regeneration, and that motor axons are sensitive to Wld^s expression. We provide the first minute-by-minute account of the destruction speed and synchrony of individual motor axons in a live intact vertebrate animal. Moreover, using overexpression and loss-of function approaches we characterize macrophage recruitment to injured nerves and demonstrate that macrophage recruitment and function occurs independently of Schwann cells. Finally, we uncover a previously uncharacterized plasticity of macrophage behavior as they interact with injured nerves.

Materials and Methods

Zebrafish Genetics and Transgenes

All transgenic lines are maintained in the Tubingen or TLF genetic background and raised as described (Mullins et al., 1994). For the majority of the experiments described

the *(Tg(mnx1:GFP)ml2* (Flanagan-Steet et al., 2005) (ZFIN ID: ZDB-ALT-051025-4) and the *Tg(Xla.Tubb:DsRed)zf148* (Peri and Nüsslein-Volhard, 2008) (ZFIN ID: ZDB-ALT-081027-2) lines were used to label spinal motor nerves, and the *Tg(spi1:Gal4,UAS:EGFP)zf149* (ZFIN ID: ZDB-ALT-081027-3) transgene to label leukocytes, including macrophages (Peri and Nüsslein-Volhard, 2008). *Sox10*$^{-/-}$ (*colorless)* mutants (Dutton et al., 2001; Kelsh et al., 1996a) were used in Figure 7. The *Tg(mnx1:Wld*^{s}*-GFP p152)* line was generated by microinjection of *mnx1:Wld*^{s}*-GFP* plasmid DNA as previously described (Thermes et al., 2002). All zebrafish work conducted in accordance with IACUC regulatory standards.

Stochastic Cell Labeling

Axons were stochastically labeled by microinjection of 33pg *mnx1:dsRed* DNA at the 1 cell stage as previously described (Thermes et al., 2002).

Plasmid Construction

Standard molecular biology methods were used to generate the *mnx1:Wld*^{s}*-GFP* plasmid in the with Isce-I meganuclease sites for DNA injection. The Wld^{s} gene was a kind gift from Dr. Milbrandt, and was cloned from the pcDNA3 vector using the flanking BamHI sites into the pBluescript vector. To make a C-terminally tagged Wld^{s} construct the Wld^{s} C-terminal end was mutagenized before the stop codon to engineer in a NheI site (Stratagene Quikchange II XL site directed mutagenesis kit). eGFP was then cloned into the NheI site. A EcoRI/XbaI digestion was used to clone Wld^{s}-eGFP into pCS2+, and then using the Gateway system (Hartley et al., 2000) Wld^{s}-eGFP was moved into the

pIsce-I expression plasmid downstream of the mnx1 promoter in between the SpeI and XbaI sites.

Wld^S-GFP Site Directed Mutagenesis Primers:

5'ACCATTCCACTTTGGCTAGCTCATCACCATCACC3' (forward) and

5'GGTGATGGTGATGAGCTAGCCAAAGTGGAATGGT3' (reverse)

were used to engineer in a NheI site before the Wld^S stop codon.

Nerve Transection

Nerve injury was performed using a MicroPoint Computer-Controlled ablation system (Photonic Instruments, St. Charles IL) consisting of a nitrogen-pumped dye laser (wavelength 435 nm) controlled by MetaMorph version 7.7 or by Slidebook version 5.0. Ablation laser settings on either software package ranged from power 55-72 depending on the age of the cumerin dye. One to four motor nerves per larva in hemisegments 10-16 were transected in all experiments, except in Figure 4 where 28 motor nerves (hemisegments 5-33) were transected per larva. To transect nerves a thin rectangular ROI was drawn digitally over the image of the nerve ~20 µm from the spinal cord exit point in either Slidebook or Photonics Instrument MicroPoint, and the nerve was laser pulsed precisely within that ROI in ~20 second intervals until all axons in the nerve appeared transected, whereby axonal fluorescence did not refill the ROI in >10 seconds.

Live Imaging

Larvae at 5 dpf were mounted in MatTek glass bottom culture dishes in 1.5% low melt SeaPlaque agarose prepared with Ringers plus Tricaine (0.016% tricaine). Images were acquired on an Olympus IX 71 or 81 microscope equipped with a Yokogawa CSU 10 scan head combined with a Hamamatsu EMCCD camera (model C9100-13, Bridgewater, NJ). Acquisition and hardware were controlled by MetaMorph version 7.7 or Slidebook version 5.0, respectively. Diode lasers for excitation (488 nm for GFP and 561 nm for dsRed) were housed in a Spectral Applied Research launch (Richmond Hill, Ontario). Image stacks for timelapse movies were acquired every 5-10 minutes, typically spanning 60-75 µm at 1 µm intervals, with an Olympus 60X, 1.2 NA UPlanSApo water immersion objective. The gain for all images captured was set at 191, resolution was 512 x 512 pixel resolution, and image capture time for nerves was between 150-300 ms (50-100 ms for cell bodies). For imaging over 2 hours, Immersol was used instead of water. GFP emission filter: ET 525/50, lot no. 119342, mCherry emission filter: ET 630/75, lot no. 200406.

Image Processing

Image stacks were compressed into maximum intensity projections (MIP) in their respective acquisition software package. MIPs were exported and gamma adjusted to 0.5 in ImageJ for increased visibility, color assigned by acquisition wavelength, and analyzed. Brightness, contrast, and color levels were adjusted for maximal visibility in Adobe Photoshop CS4.

Electron Microscopy

Embryos at 5 dpf were fixed in 6% glutaraldehyde in either 0.1 M cacodylate or phosphate buffer pH 7.2-7.4 for at least 1 hour at room temperature, and used immediately or stored for up to several days at4°C in the fixative. Head, yolk sac, yolk extension and most of the tail fin were removed within the first minutes of fixation to allow better penetration of the fixative. Tails were post-fixed in 2% OsO4 in the same buffer, en-bloc stained with saturated aqueous uranyl acetate for 2 hours, and embedded in Epon 812. Sections were cut in an ultra-microtome Leica Ultracut R (Leica Microsystem, Austria) using a Diatome diamond knife (Diatome Ltd. CH-2501 Biel, Switzerland), stained in lead citrate solution and examined in a Phillips 410 electron microscope (Philips Electron Optics, Mahwak, NJ) equipped with a Hamamatsu C4742-95 digital imaging system (Advanced Microscopy Techniques, Chazy, NY).

Behavioral Assays, Video Recording, and Behavioral Analysis

Acoustic startle responses were elicited, recorded, and measured as previously described (Burgess et al., 2009), with the following modifications. To record acoustic startle responses, high-speed video images were recorded using a Motion Pro camera (Redlake, Tucson, AZ) at 1,000 frames per second, and with 512 x 512 pixel resolution, using a 50 mm macro lens. Behavioral analysis was carried out with the FLOTE software package to determine initiation and the kinematic properties of acoustic startle responses (Burgess and Granato, 2007; Burgess et al., 2009). Acoustic startle stimuli were provided by a small vibrational excitor (Bruel and Kjaer, Norcross, GA), with 3 ms duration, 1000 Hz waveforms, of approximately 150 m/s^2. Stimulus intensity was calculated by measuring

the approximate displacement of the testing arena due to vibration. To evaluate acoustic startle behavior images were recorded 30 ms prior to and 90 ms following the delivery of the acoustic stimulus. All acoustic startle experiments were performed in a 4x4 testing arena so larvae could be tracked and analyzed individually. The 4x4 testing grids were laser-cut from acrylic by Pololu Corporation (Las Vegas, Nevada) and then glued to a circular acrylic base plate (56 mm diameter, 1.5 mm thick; Pololu Corporation) with thin acrylic cement (Weld-On #3, IPS Corporation). The base plate was affixed to the inside of a 6 cm Petri dish lid with acrylic cement (Weld-On #16, IPS Corporation). The Petri dish lid was then attached to a metal ring with modeling clay, and the metal ring was connected to the vibrational excitor by a titanium rod (Burgess and Granato, 2007; Burgess et al., 2009). For image capture purposes, a 96 bulb infrared LED array (IR100 Illuminator removed from housing, YYtrade Inc.) was positioned below the testing arena. A three mm-thick sheet of white acrylic, positioned approximately 3 cm below the testing arena, diffused the IR light. A white LED bulb (PAR38 LED light, LEDlight.com) was positioned above the testing arena to illuminate the testing arena with white light.

Results

Zebrafish spinal motor nerves as a model for injury induced Wallerian Degeneration

To monitor the events during nerve degeneration in real time we imaged zebrafish spinal motor nerves between five and seven days post fertilization (dpf) following complete nerve transection. We used a pumped dye laser (MicroPoint, Andor Technology) to transect individual, *Tg(mnx1:GFP)* positive motor nerves within the first ~20 µm of their peripheral trajectory (Figure 1A, B, (Flanagan-Steet et al., 2005). Zebrafish motor nerves consist of ~70 motor axons (Myers, 1985; Westerfield et al., 1986b), of which the large diameter axons are myelinated by Schwann cells, while the small diameter axons exhibit little or no myelination (Figure 1F). To visualize individual axons in the context of the entire nerve we stochastically labeled individual motor neurons using *mnx1:dsRed* (for details, see Material and Methods). Individual motor axons form primary, secondary and tertiary branches that synapse with the underlying muscle fibers, and also form myotendinous junctions along hemisegment boundaries (Figure 1B, (Myers et al., 1986; Westerfield et al., 1986b; Zhang et al., 2004). Time-lapse analysis of uninjured motor nerves revealed occasional and short-lived (<20 minutes) filapodial extensions and retractions of less than 11 µm (Movie 1). Importantly, the overall anatomy and branching pattern of individual axons and nerves was stable over several hours and even days, characteristic for mature peripheral nerves.

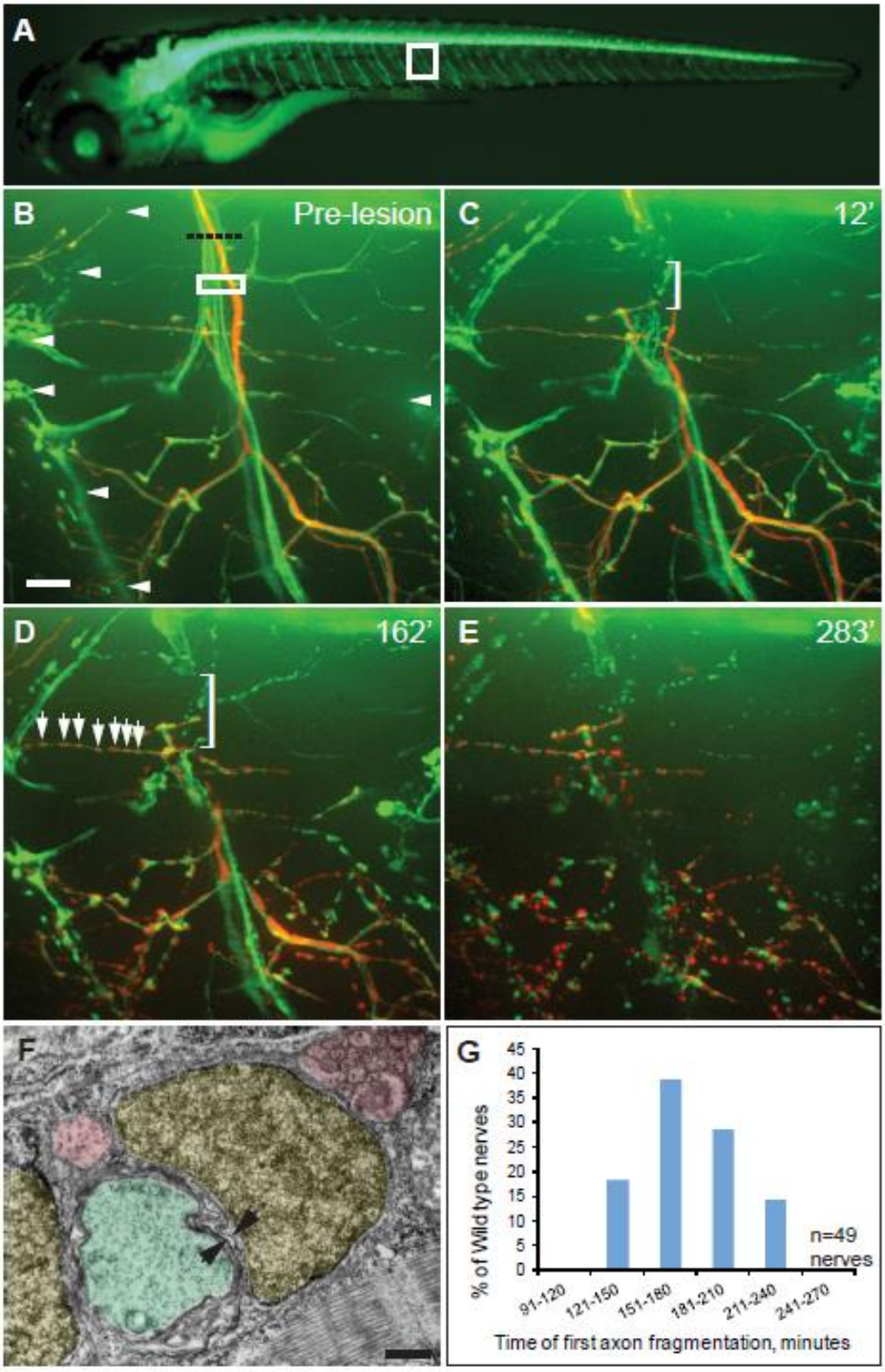

A
B
Pre-lesion
C
12'
D
162'
E
283'
F
G
% of Wild type nerves
45
40
35
30
25
20
15
10
5
0
n=49
nerves
91-120
121-150
151-180
181-210
211-240
241-270
Time of first axon fragmentation, minutes

Figure 1. Nerve degeneration in zebrafish. (A) 5 dpf *Tg(mnx1:GFP)* larva expressing GFP in spinal motor neurons and their axons. White box outlines a single motor nerve. (B) Spinal motor nerve magnified from white box in (A) with single cell *mnx1:dsRed* labeling. White rectangle indicates area of laser axotomy; arrowheads point to the nerve extending along the hemisegment boundary. (C) Following transection a gap forms in the nerve (bracket). (C, D) Axon fascicles retract both proximally and distally from the lesion site (bracket) until fragmentation starts (D, arrows). Eventually, the entire distal nerve fragments, and the debris is gradually cleared (E). Scale bar is 10 μm. (F) Electron micrograph of a cross section through a trunk motor nerve at the depth of the dashed line in (B). Schwann cells are shaded yellow, small diameter axons are shaded pink, and large diameter axons are shaded green. Arrows point to myelin sheath. Scale bar is 500 nm. (G) Quantification of nerve fragmentation onset from 49 nerves in 18 animals.

We first documented the morphological changes following complete nerve transection (Figure 1, see also Movie 2). Immediately following lesion the proximal and distal nerve fascicles sprang apart, resulting in a gap at the lesion site (Figure 1C). Over the next 120-240 minutes the proximal and distal nerve stumps continued to retract until the distal portion of the nerve began to fragment rapidly (Figure 1D, quantified in G). Interestingly, once initiated, fragmentation occurred along the entire length of individual axons within minutes (Figure 1D, Figure 2 A-F). Individual axons within the transected nerve initiated fragmentation at different times, independent of axon diameter or myelination (Figure 2 C-F). Eventually, the entire nerve fragmented (Figure 1E), and over the next 24 hours axonal debris was gradually removed (see below, Figure 4B, C). Analysis of 49 transected nerves in 18 animals revealed that spinal motor nerve degeneration occurs with stereotyped and quantifiable parameters. As shown in Figure 1G, axonal fragmentation is first detected between 121 and 240 minutes, with the majority of nerves starting to fragment between 151 and 210 minutes post transection.

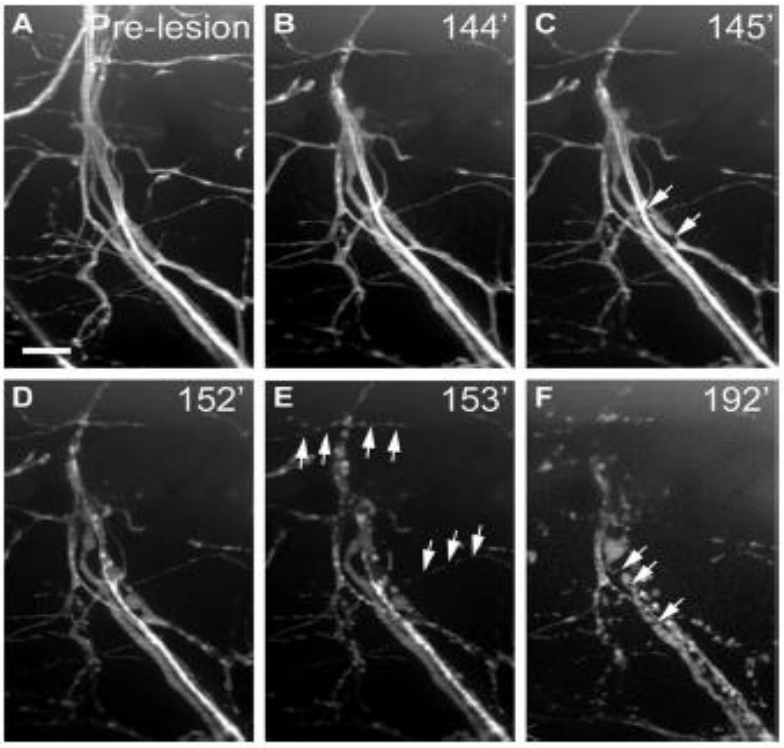


Figure 2. Individual axons degenerate independently, with sub-minute timing, and succession of fragmentation is independent of axon diameter. (A) Pre-lesion image of motor nerve in 5 dpf *Tg(mnx1:GFP)* larva. (B) All axons are intact the minute before the first axon, of large diameter, begins to fragment (C, arrows). Individual axons continue to fragment within the nerve (D) and after one minute several small diameter axons fragment (E, arrows), followed later by another large diameter axon (F, arrows). Scale bar is 10 μm.

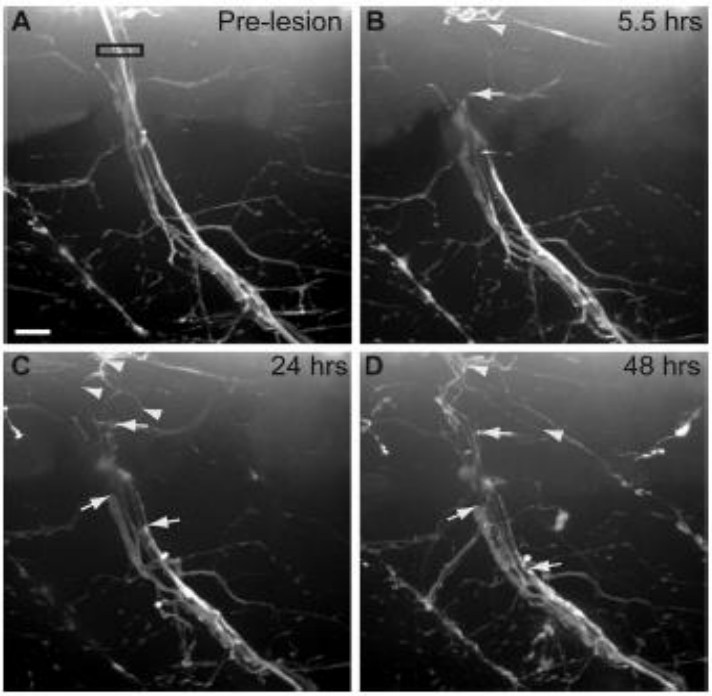


Figure 3. Axonal expression of Wld^{s} delays Wallerian degeneration.

(A) Pre-lesion image of a *Tg(mnx1:Wld^{s}-GFP); Tg(mnx1:GFP)* expressing nerve. Black box indicates area of laser axotomy. (B) Although completely transected, the distal portion of *Tg(mnx1:Wld^{s}-GFP); Tg(mnx1:GFP)* expressing nerve remains intact. Arrowhead indicates proximal nerve stump; arrows indicate most proximal end of distal nerve fascicles. Distal nerve remains intact 24 hpt (C) and 48 hpt (D), while axons continue to retract from lesion site (arrows). Some axons from the proximal stump have begun to regrow (arrowheads). Scale bar is 10 μm.

In all species tested, expression of the Wallerian Degeneration Slow protein (Wld^S) significantly delays the onset of axon fragmentation through an axon-autonomous mechanism, consistent with an evolutionarily conserved, Wld^S-sensitive axonal destruction program (Adalbert et al., 2005b; Araki et al., 2004a; Hoopfer et al., 2006b; Lunn et al., 1989a; MacDonald et al., 2006b; Martin et al., 2010; Raff et al., 2002; Wang et al., 2001a). Transient expression of Wld^S in individual zebrafish sensory and CNS axons provides neuroprotection (Feng et al., 2010; Martin et al. 2010), however, neither transient nor transgenic Wld^S models for zebrafish motor neurons have been reported. Therefore, we generated several stable transgenic lines expressing high levels of GFP tagged Wld^S under the control of the motor neuron specific *mnx1* promoter. Motor nerves expressing *Tg(mnx1:Wld^S-GFP)p152* are morphologically indistinguishable from those in wild-type animals (Figure 3A). Following laser mediated transection, Wld^S-GFP expressing nerves do not degenerate and instead remain intact for up to 8 days (n=25 nerves; Figure 3B-D compared to Figure 1D, E). Thus, stable transgenic expression of Wld^S in zebrafish motor neurons efficiently delays injury induced Wallerian degeneration. Combined, these data demonstrate that following transection zebrafish spinal motor axons degenerate through a Wld^S sensitive process with morphological hallmarks characteristic of Wallerian degeneration.

Functional recovery of transected motor nerves

In vertebrates, peripheral axons have retained their capacity for regrowth and frequently achieve functional regeneration. Regrowth of individual sensory and CNS axons has been documented in larval zebrafish (Bhatt et al., 2004; Martin et al., 2010;

O'Brien et al., 2009; Reimer et al., 2009; van Raamsdonk et al., 1998; Wyatt et al.), yet a time course of functional recovery of transected peripheral nerves has not yet been reported. *Tg(mnx1:GFP)* nerves containing a small number of *mnx1:dsRed* positive axons (1-5) were transected as before and repeatedly imaged over a time course of up to five days (Figure 4A-D). 9.5 hours post transection (hpt) the entire nerve portion distal to the transection site had fragmented, leaving behind only axonal debris (Figure 4B). Importantly, the somata of axotomized *mnx1:dsRed* positive motor neurons survived (Figure 4E-G, *n*=19/19), and within 24 hpt these motor neurons re-extended axons, along with GFP^{+} axons, past the lesion site into the ventral myotome (Figure 4C, *n*=29/52). Regenerating axons regrew through the center of the hemisegment, following the precise trajectory originally established during development (Westerfield et al., 1986b). Within 48 hpt, GFP and dsRed positive axons extended and re-established complex branches throughout the ventral myotome and along hemisegment boundaries (Figure 4D, *n*=23/37).

We next asked whether these regrown axons restore functionality to their muscle targets. To address this we utilized a well-established and quantifiable behavioral assay, the startle response (Burgess and Granato, 2007; Kimmel et al., 1974; Liu and Fetcho, 1999). The startle response is characterized by a fast, 'C'-shaped turning movement, followed by a short swimming episode, and its performance critically depends on the simultaneous and unilateral activation of trunk muscle by spinal motor nerves (Fig. 3h and Eaton and Hackett, 1984; Liu and Westerfield, 1988; Liu and Fetcho, 1999). Before nerve transections *Tg(mnx1:GFP)* larvae performed startle responses with stereotypic

kinematic parameters, including a characteristic initial head turning angle of ~130^{o} towards the right or left side (Figure 4N, see also Movie 3A, 4A). In each larva we spared the four anterior nerves, but transected the remaining 28 posterior spinal motor nerves innervating the right trunk and tail muscles (see Material and Methods for details). At three, 24 and 48 hpt we assayed the ability of these larvae to perform rightward and leftward startle responses. At three and 24 hpt the head turning angles of rightward startle responses and overall swimming distances were dramatically reduced (Figure 4I, N, O, see also Movie 3B, 4B). In contrast, by 48 hpt these parameters had reached pre-lesion levels, suggesting that regrowing axons restored functionality to their muscle targets (Figure 4J, N, O; see also Movie 3C, 4C; *n*=7 larvae). Importantly, head turning angles of leftward startle responses at all time points were indistinguishable from those recorded prior to transection (Figure 4K-N; see also Movie 4A-C; *n*=7 larvae). Thus, following transection spinal motor neurons survive, re-extend axons along their original trajectories, and restore functionality to their muscle targets.

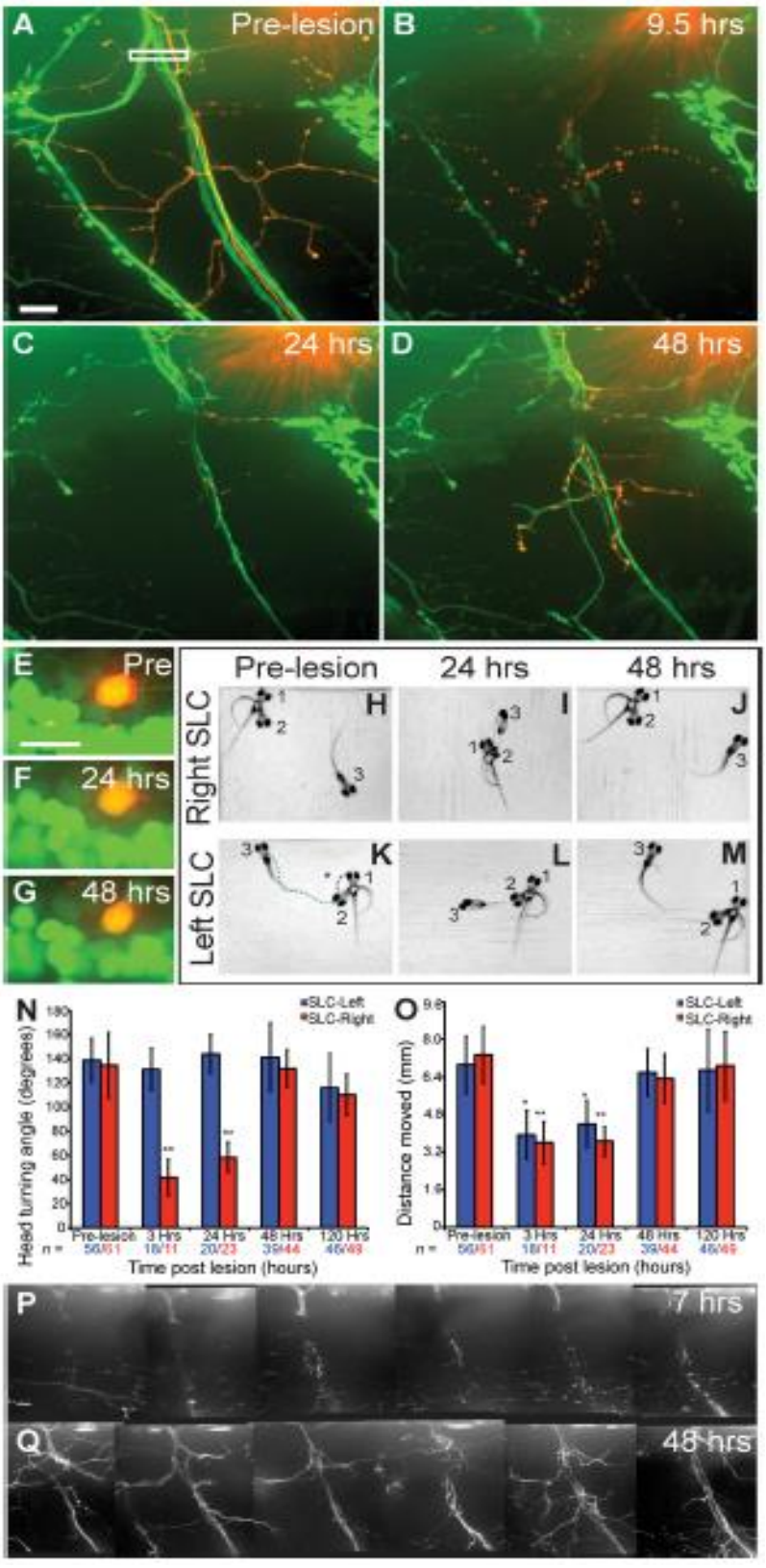


Figure 4. Functional regeneration of transected motor nerves. (A) Pre-lesion nerve in *Tg(mnx1:GFP)* with *mnx1:dsRed* co-labeled axons. White box indicates area of laser axotomy. (B) At 9.5 hpt nerve has completely fragmented. (C) At 24 hpt GFP^+ and $dsRed^+$ axons have regrown into the ventral myotome and the hemisegment boundaries. (D) At 48 hpt axons have extended throughout the ventral myotome and branched. (E-G) $DsRed^+$ and GFP^+ somas remain intact 24 and 48 hours after axotomy. Scale bar is 10 µm. (H-M) Composites from high speed movies (1,000 frames/second) document startle performance (SLC), prior (position 1) to acoustic stimulation, at maximum C-bending angle (position 2), and 90 ms after stimulation (position 3). Asterisk indicates initial head turning angle, quantified in (N), and green dotted line indicates swimming distance, quantified in (O). (N, O) Quantification of SCL performance, measuring the head angle (N) and swim distance (O), comparing turns to the left (control side, blue), and right (experimental side, red). *n*= number of rightward or leftward startle responses analyzed. *p<0.001 vs SLC-Leftward Pre-lesion, **p<0.001 vs SLC-Rightward Pre-lesion.

Macrophages arrive at the lesion site before axonal fragmentation

Having established a reliable system for visualizing nerve degeneration and regeneration, we next examined macrophage behavior in response to nerve injury. Following insult axons fragment in a stereotyped manner leaving behind cellular and membraneous debris, which is cleared by Schwann cell and macrophages (reviewed: Beuche and Friede, 1984, 1986; Lubinska, 1977; Vargas and Barres, 2007; Waller, 1849). Macrophages have been reported to accumulate at the injured nerve only after fragmentation, and their recruitment is thought to depend on signals released from Schwann cells (Avellino et al., 1995; Banner and Patterson, 1994; Hirata and Kawabuchi, 2002; Lunn et al., 1989a; Monaco et al., 1992; Perry et al., 1987; Shamash et al., 2002; Stoll et al., 1989a; Stoll et al., 1989b; Subang and Richardson, 1999; Tofaris et al., 2002; Vargas and Barres, 2007). Despite the significance of their proposed roles, when macrophages first arrive at the lesion and how they interact with injured nerves is not well documented, mainly due to the difficulties of continuously imaging inside live, intact vertebrate animals.

To monitor macrophage behavior in response to nerve transection *in vivo* and in real time we simultaneously imaged motor nerves using the *Tg(Xla.Tubb:DsRed)* transgene, and spi1^{+} immune cells using the *Tg(spi1:Gal4,UAS:EGFP)* transgene (Peri and Nüsslein-Volhard, 2008). In zebrafish, spi1 (also known as Pu.1) promotes the differentiation of macrophages from their myeloid precursors (Rhodes et al., 2005), and the *Tg(spi1:Gal4,UAS:EGFP)* line has previously been shown to mark derivatives of the leukocyte linage, including microglia in the brain, as well as early macrophages in the

trunk (Peri and Nüsslein-Volhard, 2008). Moreover, the appearance and behavior of spi1:Gal4,UAS:EGFP expressing cells that responded to nerve injury in the trunk (see below) were identical to those previously reported for zebrafish macrophages

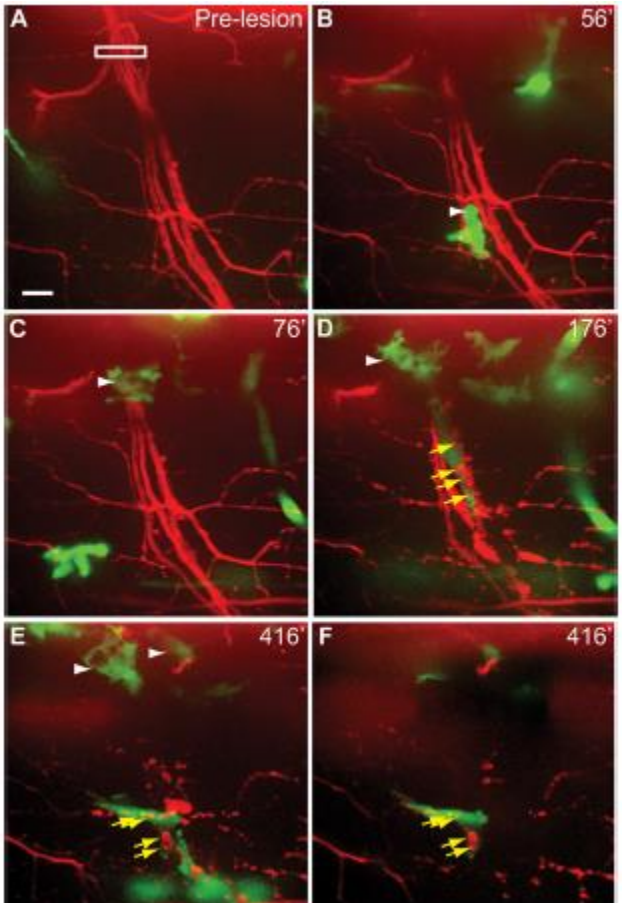


Figure 5. Recruitment and function of macrophages following nerve injury. (A) Pre-lesion image in *Tg(X1a.Tubb:DsRed)*; *Tg(spi1-Gal4,UAS-GFP)* larva. White box indicates area of laser axotomy. (B) macrophages (arrowhead) arrive and contact the lesioned distal nerve. (C) Additional macrophages (arrowhead) are recruited to the lesion site. (D) Upon onset of nerve fragmentation, macrophages phagocytose distal axon debris (arrows). (E) Hours after lesioning, macrophages remain at both the lesion site (arrowheads) and the distal nerve where they phagocytose debris (arrows). (F) Projection of eight focal z-planes from (E), totaling 8µm deep, show red axonal debris within green macrophages. Scale bar is 10 µm.

(Herbomel et al., 1999), and thus we will refer to these cells as macrophages. Prior to nerve transection the majority of macrophages were within blood vessels, and although a small number of *spi1:Gal4,UAS:EGFP* expressing cells localized outside the vasculature, they were never observed along motor nerves (Figure 5A). Analysis of 49 transected nerves revealed that in over 80% of cases macrophages left the vasculature and arrived at nerves within the first 120 minutes post lesion, which is the earliest time point we

observed the onset of axonal fragmentation (Figure 5B, C, see also Movie 5). Moreover, in 69% of cases the first macrophages arrived within 60 minutes, about 60-120 minutes before the onset of axonal fragmentation (Table 1). Upon their arrival, macrophages preferentially localized to the distal nerve stump immediately adjacent to the lesion site, where some of these macrophages became stationary and assumed an ovoid morphology, though their membranes continued to ruffle (Figure 5C). As soon as axons in the distal nerve began to fragment, macrophages infiltrated the fragmenting nerve and began to phagocytose axonal debris (Figure 5D-F, see also Table 1). Thus, macrophages arrive at the injury site long before nerve fragmentation, and with the onset of axonal fragmentation, macrophages enter the nerve and begin to phagocytose nerve debris.

We next asked whether potential damage caused during laser axotomy to neighboring tissue, such as muscle fibers, might influence macrophage recruitment. To address this we laser damaged a small area of a muscle fiber within ~10-15 μm of the nerve (same focal plane) without visibly damaging the nerve (Figure 6A). Macrophages readily infiltrated the myotome (Figure 6B, see also Movie 6), specifically targeted the damaged muscle fiber, and eventually left the area without invading the nerve (Figure 6C-F, n=4/4; compared to Figure 5). Thus, injury to nearby muscle cells does not trigger extensive cell contacts between macrophages and the nerve, suggesting that macrophages recruitment and invasion into the distal nerve following transection likely occurs in response to signals released by nerve constituents such as the motor axons, perineural glia, and/or Schwann cells.

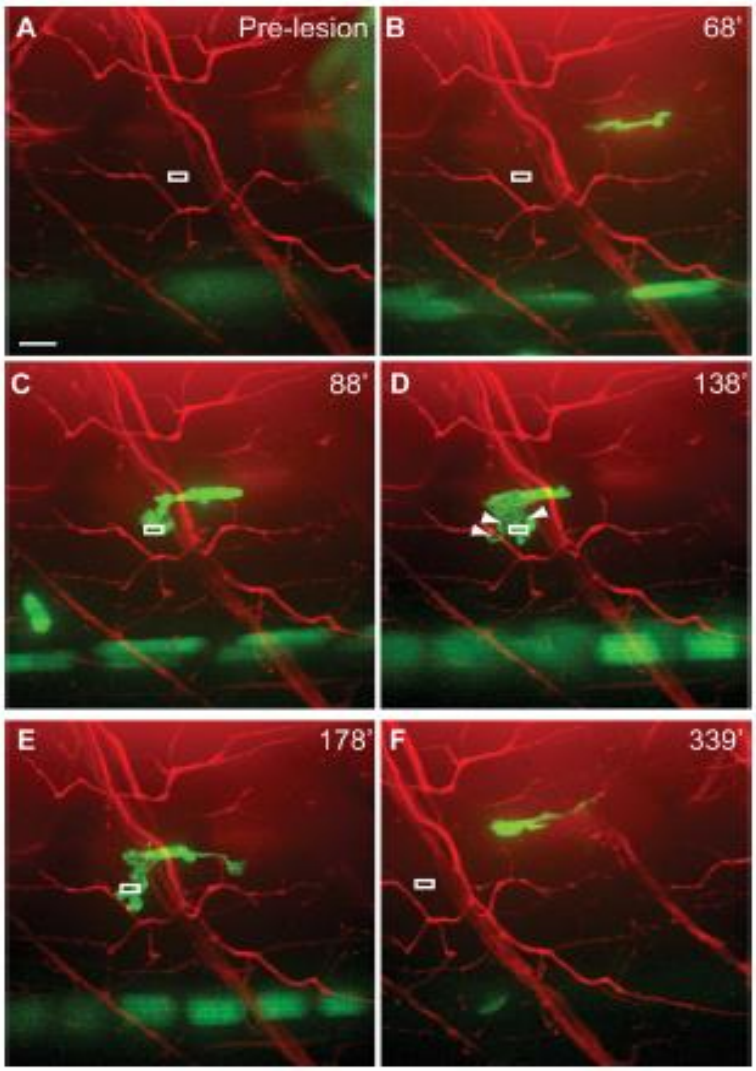


Figure 6. Recruitment and function of macrophages following muscle injury. (A-C) Image sequence shows that upon lesion to nearby muscle tissue, macrophages migrate directly to the lesion site without investigating the nerve. (A) Pre-lesion image of *Tg(X1a.Tubb:DsRed); Tg(spi1-Gal4;UAS-GFP)* larva. White box indicates area of muscle lesion (muscle cells are unlabeled), at the same focal plane of the nerve. (B) A macrophage migrates directly to muscle lesion site (C). (D, E) macrophages remain at muscle lesion, and presumably phagocytose muscle cell debris (unlabeled), evidenced by vacuoles (arrowheads). (F) Eventually, macrophages exit the lesion site. Scale bar is 10 μm.

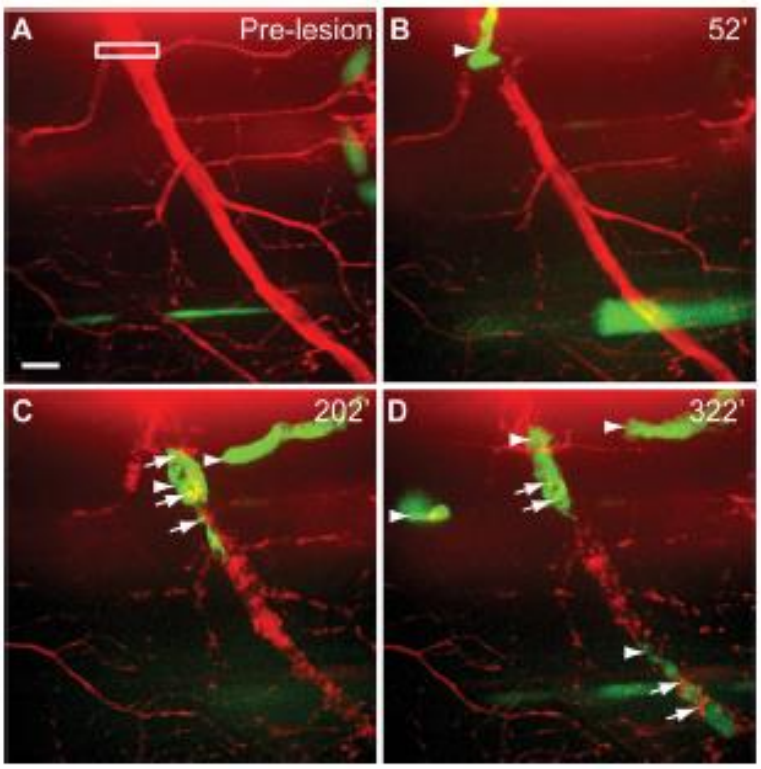


Figure 7. Macrophage recruitment to injured nerves occurs independently of Schwann cells.

(A) Pre-lesion image of *Tg(X1a.Tubb:DsRed); Tg(spi1-Gal4,UAS-GFP)*; *sox10*$^{-/-}$ larva. White box indicates area of laser axotomy. (B-D) macrophages (arrowhead) are recruited to the nerve lesion site and phagocytose distal nerve debris following the onset of fragmentation (arrows). Scale bar is 10 µm.

Macrophage behavior and recruitment to injured motor nerves is independent of Schwann cells and axonal Wld^{s} expression

Following nerve injury, macrophage recruitment and activation are thought to be triggered by reciprocal interactions between macrophages and Schwann cells (Banner and Patterson, 1994; Shamash et al., 2002; Subang and Richardson, ; Tofaris et al., 2002). However, injury induced nerve degeneration in animals with a genetic ablation of all Schwann cells has not been examined. We therefore utilized *sox10*$^{-/-}$ (*colorless*) mutants, which lack all Schwann cells (Dutton et al., 2001; Kelsh et al., 1996a). In these animals, motor axons develop without delay and at 5 dpf their motor nerves are morphologically indistinguishable from those in wild-type siblings (Figure 7A). Following transection, degeneration of *Tg(X1a.Tubb:DsRed)*; *sox10*$^{-/-}$ nerves proceeded with the same

morphological and temporal parameters we had observed in wild-type siblings (Figure 7B-D, see also Movie 7). Moreover, macrophages arrived at the nerve lesion site with similar timing and morphology as seen in wild-type larvae (Figure 7B, see also Table 1, Movie 7). Finally, macrophages also infiltrated the fragmenting distal nerve and phagocytosed axonal debris with the same time course observed in wild-type siblings (Figure 7C, D). Thus, genetic ablation of Schwann cells demonstrates that macrophage recruitment and function at injured nerves can occur independently of Schwann cells.

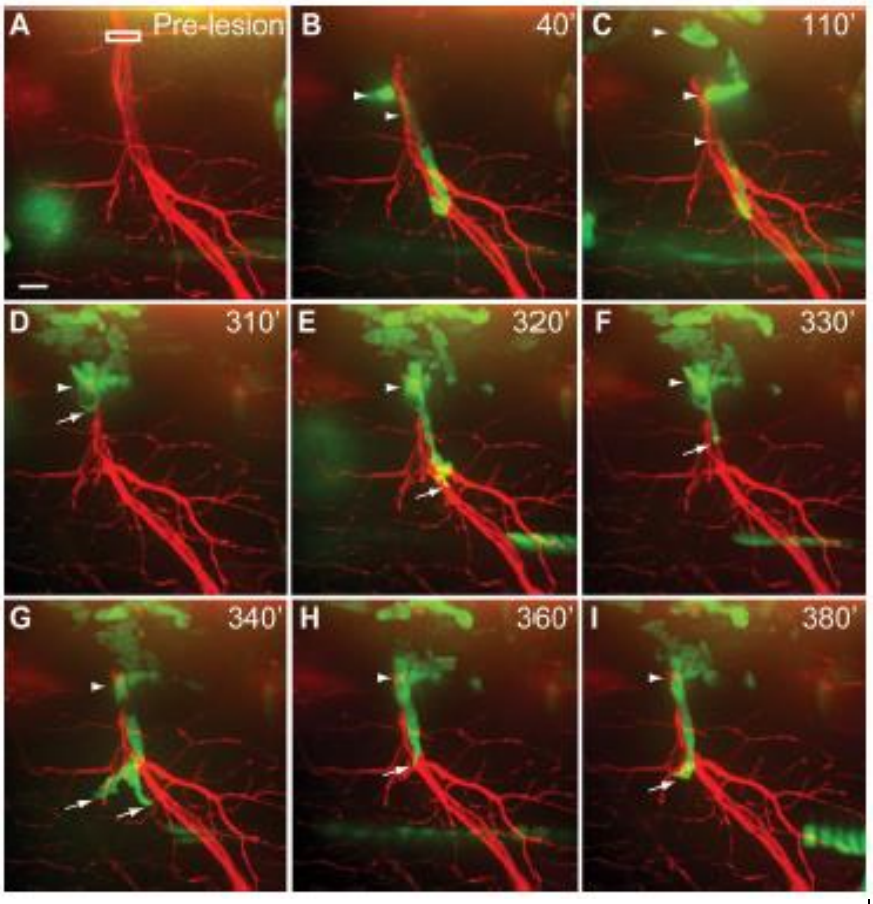


Figure 8. Macrophages are recruited to injured Wld^{s}-GFP expressing nerves and display a novel behavior.

(A) Pre-lesion image in *Tg(X1a.Tubb:DsRed); Tg(spi1-Gal4,UAS-GFP)*; *Tg(mnx1:Wld^{s}-GFP)* larva. White box indicates area of laser axotomy. (B-C) Macrophages (arrowheads) infiltrate the lesion site and accumulate at the lesioned nerve. (D-I) During first ~6.5 hours following lesion, macrophages repeatedly extend and retract processes along the injured distal nerve. Scale bar is 10 µm.

We next assessed if and to what extent macrophage recruitment and behavior are modulated by processes intrinsic to injured axons. For this we monitored macrophage behavior after injury of Wld^S-GFP expressing nerves. Prior to nerve transection macrophages behaved indistinguishably from those in *Tg(mnx1:GFP)* or *Tg(Xla.Tubb:DsRed)* animals. Following nerve transection we found that macrophages were recruited to the lesion site of Wld^S-GFP expressing nerves in similar numbers and within the same time frame when compared to wild-type nerves (Figure 8B, C; see also Table 1, Movie 8). Despite the absence of axon fragmentation of Wld^S-GFP expressing nerves, over the next ten hours following transection macrophages remained in extensive contact with the distal nerve stump immediately adjacent to the lesion site (Figure 8C-I). At irregular intervals during this time period macrophages located at the nerve stump elongated and extended a process ventrally along the distal nerve (Figure 8D-I, see also Movie 8, *n*=3/15). This 'scanning' behavior, which we did not observe in wild-type nerves prior to the onset of axonal fragmentation, occurred without detectable signs of axonal fragmentation or phagocytosis (compare Figure 5D-E with Figure 8D-I). Thus, live imaging reveals that during Wallerian degeneration macrophage recruitment is insensitive to axonal Wld^S-GFP, and describes a novel cellular behavior of macrophages when nerves fail to fragment.

Discussion

Wallerian degeneration is an early step towards functional nerve regeneration, and involves extensive cellular interactions between injured axons and multiple non-neuronal cells such as immune and glial cells. Early studies established that the environment generated by macrophages and Schwann cells is critical for successful nerve regeneration (Aguayo et al., 1981; David and Aguayo, 1981), and endpoint analysis of stained sections documented complex histological changes in Schwann cell appearance, myelin breakdown and macrophage influx following peripheral nerve injury (reviewed: Martini et al., 2008). Albeit labor intensive and complex, *in vivo* imaging of nerve degeneration in murine models has been established, yet the focus has been primarily on changes in axons (Beirowski et al., 2004; Kerschensteiner et al., 2005). Thus, despite its importance, a minute-by-minute account of the cellular interactions between injured nerves and non-neuronal cells was lacking. Moreover, which of these cellular interactions are of functional significance is largely unknown.

We have taken advantage of the transparency of the zebrafish to precisely transect mature motor nerves and to image axonal destruction and the cellular response of macrophages *in vivo* and in real time. Overall, we find that nerve degeneration proceeds with the same morphological landmarks as those reported for Wallerian degeneration in mammals (Figure 1, (Adalbert et al., 2005b; Beirowski et al., 2005; George et al., 1995; Lunn et al., 1989a; Martin et al., 2010; O'Daly and Imaeda, 1967; Raff et al., 2002; Vargas and Barres, 2007; Waller, 1849). The lag time between injury and onset of axonal degeneration is known to vary significantly between vertebrate species (reviewed in:

(Vargas and Barres, 2007), and importantly, the lag time of 121-240 minutes we observed in zebrafish remained constant as larvae aged (6-14 dpf; data not shown), consistent with previous observations that motor nerves at 5 dpf have established mature trajectories and connections (Westerfield and Eisen, 1988a). Moreover, we find that expression of Wld^{s} in motor nerves effectively delays fragmentation, as previously reported in zebrafish sensory axons and in other species (Adalbert et al., 2005b; Araki et al., 2004a; Hoopfer et al., 2006a; Lunn et al., 1989a; MacDonald et al., 2006b; Martin et al., 2010; Raff et al., 2002; Wang et al., 2001a). In addition, we demonstrate that transected motor nerves re-establish functional connections with their muscle targets (Figure 4). Taken together, this validates zebrafish motor nerves as a model for functional nerve regeneration.

Our results characterize, for the first time and with unprecedented temporal and spatial resolution, the cellular interactions between injured nerves and immune cells. Importantly, these observations combined with genetic manipulations and cell type ablation lead to several new insights into the process of Wallerian degeneration. First, the spatio-temporal progression of axonal degeneration has historically been controversial, mainly due to the limitations of visualizing individual axons during this process. Earlier studies using fixed samples reported that axonal degeneration of dorsal root ganglion axons proceeds anterogradely at a rate of ~3 mm/hour (George and Griffin, 1994b), and similar studies had correlated axonal diameter with the onset of nerve fragmentation (Lubinska, 1977, 1982), while more recent studies observed retrograde progression (Beirowski et al., 2005). By imaging individual axons within the degenerating nerve at one minute intervals, our data shows that axons and their branches fragment within less

than a minute along their entire length (Figure 2). Within a transected nerve, fragmentation of individual axons appears desynchronized and proceeds without apparent order, independent of axonal caliber (Figure 2). While species differences concerning the rate of axonal fragmentation are well documented (reviewed: Vargas and Barres, 2007), the data presented here reveals heterogeneity even amongst individual motor axons of similar length and caliber within a shared nerve, and to our knowledge provide the first minute-by-minute account of the destruction speed and synchrony of individual motor axons in a live, intact vertebrate animal.

Second, our live cell imaging demonstrates that macrophages arrive at the lesion site long before the onset of fragmentation, independent of Schwann cells. Early studies, based on fixed samples, reported that 24 hours following sciatic nerve crush macrophage counts were not increased, and that macrophages arrived at the lesion site by the second day (Bruck, 1997; George and Griffin, 1994a; Perry et al., 1987; Stoll et al., 1989b), when nerve fragmentation is well underway. Recent studies show that at 36 hours post lesion sciatic nerve fragmentation is accompanied by macrophages, yet the precise arrival of macrophages in relation to nerve fragmentation had not been established (Beirowski et al., 2004). We find that macrophages arrive at the lesion site 60-120 minutes prior to the onset of axonal fragmentation (Figure 5). Following axonal fragmentation, macrophages infiltrate the distal portion of the injured nerve and phagocytose debris, as previously observed in mammalian systems (Perry et al., 1987; Stoll et al., 1989b). To determine the necessity of Schwann cells in signaling to macrophages during Wallerian degeneration we used *sox10*$^{-/-}$ mutants which lack Schwann cells (Dutton et al., 2001; Kelsh et al.,

1996b). Schwann cells are thought to release chemokine signals that recruit immune cells to the lesioned nerve (Banner and Patterson, 1994; Shamash et al., 2002; Subang and Richardson, 1999; Tofaris et al., 2002). For example, infusion of antibodies blocking MIP-1α or macrophage chemoattractant protein-1 (MCP-1) function (Perrin et al., 2005), and genetic deletions of MCP-1 and its receptor CCR2, lead to reduced macrophage recruitment to the lesion site (Siebert et al., 2000; Toews et al., 1998). However, the role of Schwann cells on macrophage recruitment has not been determined in the complete absence of Schwann cells. We find that in the absence of Schwann cells motor axons develop normally, and upon lesion degenerate with the same morphology and kinematic parameters as observed in wild-type animals. Moreover, macrophages are recruited to the nerve lesion site with similar timing and numbers as wild-type animals, and upon axonal fragmentation also phagocytose debris (Figure 7, see also Table 1), demonstrating that Schwann cells are dispensable for macrophage recruitment and function.

Lastly, our studies uncover a previously unknown behavior of macrophages when confronted with injured nerves in which fragmentation is delayed. Axonal expression of the Wld^{s} protein not only delays nerve fragmentation, but is also thought to reduce macrophage recruitment to the lesioned nerve (Lunn et al., 1989a). Live imaging reveals that macrophages are robustly recruited to injured Wld^{s} expressing nerves with similar timing and numbers as to wild-type nerves (Figure 8, see also Table 1). Although we cannot exclude the possibility that macrophages were attracted by damage to surrounding tissues or by axonal debris generated during laser transection, we observed macrophage recruitment in 15/15 transected nerves in which neither axonal fragmentation nor

phagocytosis of axonal material by macrophages was detectable (Figure 5E compared to Figure 8C, G, I).

In the first ~10 hours following transection, macrophages maintain extensive contact with the Wld^S expressing nerve stump, and exhibited a novel 'scanning' behavior, repeatedly extending and retracting a process along the distal nerve (Figure 8). This behavior was distinct from macrophage behavior following wild-type axon fragmentation. While our observations are consistent with the idea that the prolonged presence of macrophages is insufficient to trigger axonal fragmentation, they also suggest that macrophage behavior is modulated by nerve integrity. While the absence of axonal fragmentation does not abrogate macrophage recruitment to an injured nerve, it alters 'on site' macrophage behavior. This is somewhat reminiscent of axonal injury in the CNS, where resident microglia migrate with some delay to the lesion site, but for unknown reasons exert limited phagocytic activity (reviewed: Barron, 1995; Cui et al., 2009; Lawson et al., 1994). This low rate of phagocytic activity contributes to the slower Wallerian degeneration rate in the CNS, compared to the PNS, although the rates of axonal fragmentation in the PNS and the CNS are comparable (George and Griffin, 1994b; Stoll et al., 1989a), and thus cannot account for the difference in phagocytic behavior. In contrast, the 'scanning' behavior we observe is only exhibited in the absence of nerve fragmentation. Such behavioral plasticity exhibited by leukocytes has previously not been reported and strongly suggests the existence of multiple, possibly independent signals regulating macrophage activity. A first signal attracts leukocytes to the injured nerve, while subsequent signal(s) initiate leukocyte invasion and phagocytosis of the

distal nerve. While future studies are required to identify these signals, our results demonstrate the need to visualize in real time and at the functional level the complex interactions between the cell types involved in nerve degeneration and regeneration to define the molecular mechanisms that trigger and mediate these processes.

Table 1. Quantification of macrophage recruitment to peripheral nerves following nerve transection or muscle lesion.

	Wild Type	**Wld^{s} + nerves**	***sox10*$^{-/-}$**	**Muscle**
% of macrophages arriving within 60 min post lesion	69 (n=49)	86 (n=40)	86 (n=7)	50 (n=4)
% of macrophages arriving within 120 min post lesion	86 (n=49)	100 (n=40)	86 (n=7)	100 (n=4)
Avg # of macrophages / hemisegment post fragmentation	3 (n=36)	3 (n=14)	3 (n=7)	-
Median # of macrophages / hemisegment post fragmentation	3 (n=36)	4 (n=14)	4 (n=7)	-
Max # of macrophages / hemisegment post fragmentation	8 (n=36)	6 (n=14)	5 (n=7)	-

Table indicates the number and timing of macrophage recruitment to peripheral nerves following nerve transection in 5 dpf Wild Type larvae (*Tg(X1a.Tubb:DsRed)*; *Tg(spi1-Gal4,UAS-GFP)*), Wld^{s}+ larvae (*Tg(mnx1:Wld^{s}-GFP); Tg(mnx1:GFP)*, and *Sox10*$^{-/-}$ larvae *(Tg(X1a.Tubb:DsRed); Tg(spi1-Gal4,UAS-GFP)*; *sox10*$^{-/-}$), or following muscle lesion in wild type larvae.

Chapter 3:

Schwann cells and DCC direct regenerating motor axons towards their original path

The data in Chapter Two have been published in the *Journal of Neuroscience*.

Allison F. Rosenberg, Jesse Isaacman-Beck, Clara Franzini-Armstrong, and Michael Granato

Abstract

Following complete nerve transection, a major challenge for regenerating peripheral axons is to traverse the injury site and navigate towards their original trajectory. Denervated Schwann cells distal to the lesion site secrete factors promoting axonal growth and serve as an axonal substrate, yet whether Schwann cells also actively direct axons towards their original trajectory is unclear. Using live cell imaging in zebrafish we visualize for the first time how in response to nerve transection distal Schwann cells change morphology as axons fragment, and how Schwann cell morphology reverses once regenerating growth cones have crossed the injury site and have grown along distal Schwann cells outlining the original nerve path. In mutants lacking Schwann cells, regenerating growth cones extend at rates comparable to wild type, yet frequently fail to cross the injury site and instead stray along aberrant trajectories. Providing growth permissive yet Schwann cell-less scaffolds across the injury site was insufficient to direct regenerating growth cones towards the original path, providing compelling evidence that

denervated Schwann cells actively direct regenerating axons across the injury site towards their original trajectory. To identify signals that guide regenerating axons *in vivo*, we examined mutants lacking the DCC guidance receptor. In these *dcc* mutants a significant fraction of regenerating motor axons extended along aberrant trajectories, similar to what we observe in mutants lacking Schwann cells. Thus, Schwann cell and *dcc* mediated guidance are critical early during regeneration to direct growth cones across the transection gap and onto their original axonal trajectory.

Introduction

In the peripheral nervous system injured axons regenerate, though many aspects of this process, including how regenerating axons navigate across the lesion site towards their original synaptic targets, are not well understood. The regenerative capacity of peripheral axons varies with the severity and the type of injury (Zochodne, 2008; Brushart, 2011; Zochodne, 2012). For example, following a crush injury glial derived basal lamina remains intact, providing regenerating axons with a permissive substrate across the injury site (Cajal, 1928; Scherer et al., 1984; Nguyen et al., 2002). In contrast, complete nerve transection results in a Schwann cell and basal lamina free gap, presenting a major hurdle for regenerating axons. In rodents, removing the distal nerve stump or increasing the injury gap beyond 12 mm dramatically reduces regeneration, consistent with the idea that the distal nerve provides axons with critical signals for navigation across the gap towards the distal nerve stump and onto their original trajectory (Lundborg et al., 1981; Lundborg et al., 1982; Lundborg et al., 1986).

Following injury, regenerating axons interact with a variety of non-neuronal cells, including macrophages, fibroblasts, perineural ensheathing glia, and Schwann cells (Beuche and Friede, 1984; Perry et al., 1987; Stoll et al., 1989; Martini et al., 1990; Perry and Brown, 1992; Schröder et al., 1993; Abernethy et al., 1994; Weis et al., 1994; Parrinello et al., 2010; reviewed inBrosius Lutz and Barres, 2014), yet if and to what extent these cell types direct regenerating axons towards their original developmental path has not been determined *in vivo*. Schwann cells in particular are thought to be essential for regeneration. Denervated Schwann cells in the distal nerve stump, known as the bands of Bungner (Bungner, 1891), serve as a substrate for regenerating axons, and provide diffusible factors, including NGF, BDNF and FGF, to promote axonal outgrowth (Lundborg et al., 1986; Ide, 1996; Chen et al., 2007). However, whether Schwann cells also provide directional information to regenerating axons pioneering across the injury gap is unclear.

Here we take advantage of the genetic tractability and transparency of larval zebrafish to investigate how regenerating motor axons cross an injury-induced transection gap and select their original trajectory in live, intact animals. Using *in vivo* microscopy we compared the dynamic behavior of regenerating axons and Schwann cells in wild type larvae to mutants lacking all Schwann cells. Remarkably, the absence of Schwann cells did not impede growth cone sprouting nor axonal growth as regenerating axons extended over considerable distances. However, axons lacked directionality and traveled along ectopic trajectories. Providing Schwann cell-less axonal scaffolds across the injury site and along the original trajectory was insufficient to fully restore directionality to regenerating axons, suggesting that Schwann cells produce factors that direct

regenerating axons to their appropriate trajectory. Finally, in mutants lacking the axonal guidance receptor *dcc*, regenerating axons strayed from their original path onto ectopic trajectories, reminiscent of the phenotype observed in mutants lacking Schwann cells. We conclude that Schwann cells and *dcc* dependent signaling direct regenerating axons towards their original developmental trajectories *in vivo*.

Materials and Methods

Zebrafish Genetics and Transgenes

All transgenic lines were maintained in the Tuebingen or TLF genetic background and raised as described (Mullins et al., 1994). The *Tg(mnx1:GFP)*^{ml2} (Flanagan-Steet et al., 2005) and the *Tg(Xla.Tubb:DsRed)*^{zf148} (Peri and Nüsslein-Volhard, 2008) lines were used to label spinal motor nerves. *Tg(sox10(7.2):mrfp)*^{vu234} (Kucenas et al., 2008) and *Tg(sox10:nlseos)*^{w18} (Prendergast et al., 2012) lines were used to label Schwann cells. The *Tg(mnx1:Wld*^{s}*-GFP)*^{p152} line (Rosenberg et al., 2012) expresses the Wld^{s}-GFP protein in motor neurons. The *sox10*$^{-/-}$*/colourless (cls)*^{m241}, *erbb2*^{st50}, *erbb3*^{st48}, *dcc*^{zm130198} and *nrg1*^{z26} (Kelsh et al., 1996; Dutton et al., 2001; Lyons et al., 2005; Jao et al., 2008; Perlin et al., 2011) mutants were used. Male and female zebrafish were utilized, and all zebrafish work was conducted in accordance with IACUC regulatory standards.

Stochastic Cell Labeling

Axons were stochastically labeled by microinjection of 33pg *mnx1:DsRed* DNA at the 1 cell stage as previously described (Thermes et al., 2002). The DsRed fluorophore is

strongly expressed by 24 hpf, concomitantly with the expression of GFP in the transgenic line *Tg(mnx1:GFP)*^{ml2}.

Genotyping

Genotyping protocols for the following mutants were performed as previously described: *dcc*^{zm130198} (Jain et al., 2014); *erbb2*^{st50} and *erbb3*^{st48} (Lyons et al., 2005); and *nrg1*^{z26} (Perlin et al., 2011).

Whole-mount fluorescent in situ hybridization and Immunohistochemistry

Antisense digoxigenin labeled *dcc* RNA probes were used for *in situ* hybridization and was performed as described previously (Lakhina et al., 2012). *In situ* signals were amplified using a cyanine 5-coupled tyramide system (TSA Plus Cyanine 5 System, PerkinElmer, product number NEL745001KT). *In situ* hybridization was followed by immunohistochemistry using rabbit anti-GFP (1:400, Life Technologies) and goat anti-rabbit AlexaFluor488 conjugated secondary antibody (1:500; Invitrogen) to visualize motor neurons. Processed larvae were mounted laterally in Vectashield (Vector Laboratories) and imaged in 1 µm sections with a 20x water lens and a 60x oil-immersion lens on a Zeiss 710 confocal laser scanning microscope (LSM 710) using ZEN2010 software.

The anti-sox10 antibody was a generous gift from the Kucenas laboratory. 5 day old zebrafish larvae were fixed in 4% PFA with 0.1% TritonX-100 for 3 hours, then washed 1x5 minutes successively with PBS with 1% Triton-X100 (PBStx), deionized water with 1% Triton-X100, and 100% acetone, followed by 100% cold acetone for 10

minutes at -20°C. Then larvae were washed 3x5 minutes in PBStx, blocked in 5% goat serum/PBStx, and incubated in 5% goat serum/PBStx/1° Antibody for 1 hour at room temperature then 4°C overnight. Larvae were washed extensively with PBStx and incubated with goat anti-rabbit AlexaFluor594 conjugated secondary antibody (1:500, Invitrogen). Larvae were mounted in Vectashield (Vector Laboratories) and images were acquired with an Olympus IX81 equipped with a Yokogawa CSU 10 scan head combined with a Hamamatsu EMCCD camera (model C9100-13, Bridgewater, NJ), acquisition and hardware were controlled by Slidebook version 5.0.

Flourescent mRNA Quantification

In FIJI, fluorescent double-labeled 6 µm stacks for *dcc* mRNA and motor neuron-GFP were made into a series of maximum projection images, then processed to subtract background, and a mean filter of 1 pixel was applied. A region of interest (ROI) was drawn around the ventral spinal cord where motor neurons were labeled with GFP. mRNA levels within the ROI were counted in two ways: first, by counting the number of local maxima, and second by measuring overall fluorescence levels. This analysis was repeated in smaller ROIs drawn around individual GFP positive cell bodies.

Nerve Transection and Live Imaging

Nerve transection and live imaging was performed as previously described (Rosenberg et al., 2012).

Axon Growth Extent Quantification

Axon growth extent was quantified 48 hours post transection (hpt) using a rubric of three semi-quantitative categories, illustrated in Figure 1 N-P. Transected nerves where axons failed to regrow or did not extend through the entire length of the ventral myotome are categorized as "no/weak regeneration". Nerves with at least one fascicle that extended through the entire length of the ventral myotome are categorized as "moderate regeneration". Finally, nerves with two or more fascicles extending through the entire length of the ventral myotome are categorized as "strong regeneration".

Axon Misguidance Quantification

Misguided axons were identified at 48 hpt as axons above the lateral line that regrew in areas that were not populated by axons in pre-lesion images.

Axon Growth Rate, Schwann Cell Migration and Proliferation Quantification

Regenerating nerves and Schwann cells were imaged approximately every 10 minutes from ~6 to ~13 hpt, as described previously (Rosenberg et al., 2012). In ImageJ the scale was set on these movies to 0.192 μm/pixel, and pixel distance and aspect ratio to 1.0. Growth was measured at each hour by drawing lines over newly grown axon segments and recording the length in microns per hour. Schwann cell distance was measured using the same method, measuring from the top of the distal stump to the leading edge of the Schwann cell nucleus at each time point. Numbers of migrating and proliferating Schwann cells were counted.

Electron Microscopy

Nerves were transected as described previously in 5 dpf larvae, then larvae were removed from agarose and incubated in E3 at 28°C until fixation at 6 hpt or 48 hpt, following previous methods (Rosenberg et al., 2012). Longitudinal coronal sections through the tail segment extending for several myotomes on either side of the transected regions were cut starting from the ventral side and progressing dorsally. When the notochord was reached a group of thin sections was cut at intervals of ~12-15 μm in order to view cross sections of the nerve distal and up to the damaged sites. Cross sectional profiles of nerves were present immediately adjacent to the notochord midway along each sarcomere. Profiles on the uninjured left side of the same animals were used as control undamaged nerves.

Image Processing

Image stacks were compressed into maximum intensity projections (MIPs) in Slidebook version 5.0. MIPs were exported and gamma adjusted to 0.5 in ImageJ for increased visibility using a custom macro called "Gamma Adjustment Batch: 0.5", and color assigned by acquisition wavelength and merged using a custom macro called "Batch RG Merge," modified from a macro called "Batch RGB Merge." Both macros were deposited in the online ImageJ macro collection and can be downloaded at http://rsb.info.nih.gov/ij/macros/. Brightness, contrast, and color levels were adjusted for maximal visibility in Adobe Photoshop CS4.

Statistical Analysis

Fisher Exact T tests were performed on all applicable data sets.

Results

Live cell imaging following nerve injury reveals orchestrated cellular dynamics of axons and Schwann cells

Changes in Schwann cell morphology are a hallmark of nerve degeneration, yet the *in vivo* dynamics of this process have not been observed. We therefore fully transected individual nerves in larvae aged 5 days post fertilization (dpf) and then documented changes in Schwann cell morphology using time lapse microscopy (Figure 1A). Ventrally projecting zebrafish motor nerves consist of ~60-80 motor axons, and individual axons form branches that synapse with muscle fibers and myotendinous junctions along hemisegment boundaries (Figure 1A, B and Myers, 1985; Myers et al., 1986; Westerfield et al., 1986; Zhang et al., 2004; Rosenberg et al., 2012). At 5 dpf large diameter axons are wrapped by ~3-5 layers of myelin, roughly equivalent to that in mouse and rat during the first postnatal week (Peters and Muir, 1959; Schlaepfer and Myers, 1973; Hahn et al., 1987; Garbay et al., 2000). Motor nerves were laser transected as previously reported (Rosenberg et al., 2012) within the first ~20 µm of the peripheral nerve trajectory in larvae expressing GFP in motor neurons and RFP in Schwann cells *Tg(mnx1:GFP; sox10:mRFP)*. Prior to axon fragmentation Schwann cell membranes were elongated and closely associated with motor axons (Figure 1B, C). Once axons started to fragment, Schwann cell membranes distal to the lesion site began to reorganize, changing from a tube like morphology to a shorter, more rounded morphology containing large blebs of fluorescently labeled axonal debris (Figure 1D-H, and Movie 1). The fluorescent intensity of the encircled debris decreased over the next two hours (Figure

1E-H), consistent with the observation that axonal debris is degraded by phagocytic vesicles present in Schwann cells (Holtzman and Novikoff, 1965; Scherer et al., 1984).

To visualize changes in Schwann cell morphology as axons regenerate, we transected nerves and imaged Schwann cells and regenerating motor axons over a 48 hour time period (Figure 1I-L). By 7 hours post transection (hpt) the nerve segment distal to the transection site had fragmented, leaving behind only axonal debris and a ribbon of denervated distal Schwann cells, known as the band of Bungner (Fig. 1J Bungner, 1891). Compared to pre-lesion Schwann cell membranes (Figure 1I), these distal Schwann cell membranes appeared distended and gnarled (Figure 1E-H). Within 24 hpt motor neurons re-extended axons past the lesion site, in close proximity to the original path delineated by denervated Schwann cells (Figure 1K). By 48 hpt, regenerating axons had traversed the ventral myotome where they re-established complex branches. Concomitantly, Schwann cell membranes reverted to their thinner appearance in proximity to regenerating axons (Figure 1I-L), and their membranes re-acquired a smoother, pre-lesion like morphology (Figure 1I). Single micron z-planes (Figure 1I-L insets) demonstrate that axons extended in close proximity to the denervated distal Schwann cells, similar to what has been shown in mammalian systems (Scherer et al., 1984; Westerfield et al., 1986; Chen et al., 2007; Zochodne, 2008; Rosenberg et al., 2012). Thus, laser axotomy triggers changes in axonal and Schwan cell morphology, and live imaging reveals that these changes are highly coordinated between the two cell types, both during degeneration as well as during regeneration.

To quantify the extent of axon regeneration at 48 hpt we applied a three category rubric illustrated in Figure 1M-Q (for details see Materials and Methods). In wild type

larvae ~80% of transected motor nerves (n=85 nerves from 22 larvae) regrew successfully through the length of the ventral myotome (Figure 1Q, "strong" and "moderate" categories), and formed functional synapses (Rosenberg et al., 2012). We next wanted to determine the extent of laser axotomy induced nerve degeneration, and the degree of regeneration at the ultrastructural level. For this we performed electron microscopy at 6 hpt when most axons have fragmented, and at 48 hpt when axons have regenerated (Figure 1 and Rosenberg et al., 2012). Compared to uninjured nerves containing Schwann cells adjacent to axonal profiles separated by myelin sheets (Figure 2A, B), axotomized nerves at 6 hpt appeared disorganized at the level of the transection site, lacking discernable axonal membranes and instead containing collapsed myelin sheets (Figure 2C). Similar to intact control nerves (Figure 2D, E), transected nerves at 48 hpt contained Schwann cells adjacent to both small and large diameter axons, which had regrown past the lesion site onto their original path (Figure 2F). At this time point, the levels of myelination had not yet returned to pre-lesion levels, with only a presumptive partially formed myelin sheet detectable (Figure 6E compared to F). Thus, similar to mechanical injury, laser axotomy causes complete nerve transection at the lesion site, providing a suitable system to examine how regenerating axons navigate an injury gap devoid of Schwann cells and their associated basal lamina.

Schwann cell migration and proliferation during the early phase of axon regeneration

We next asked how individual Schwann cells respond to nerve transection. Previous studies have shown that Schwann cell migration and proliferation are critical to axon

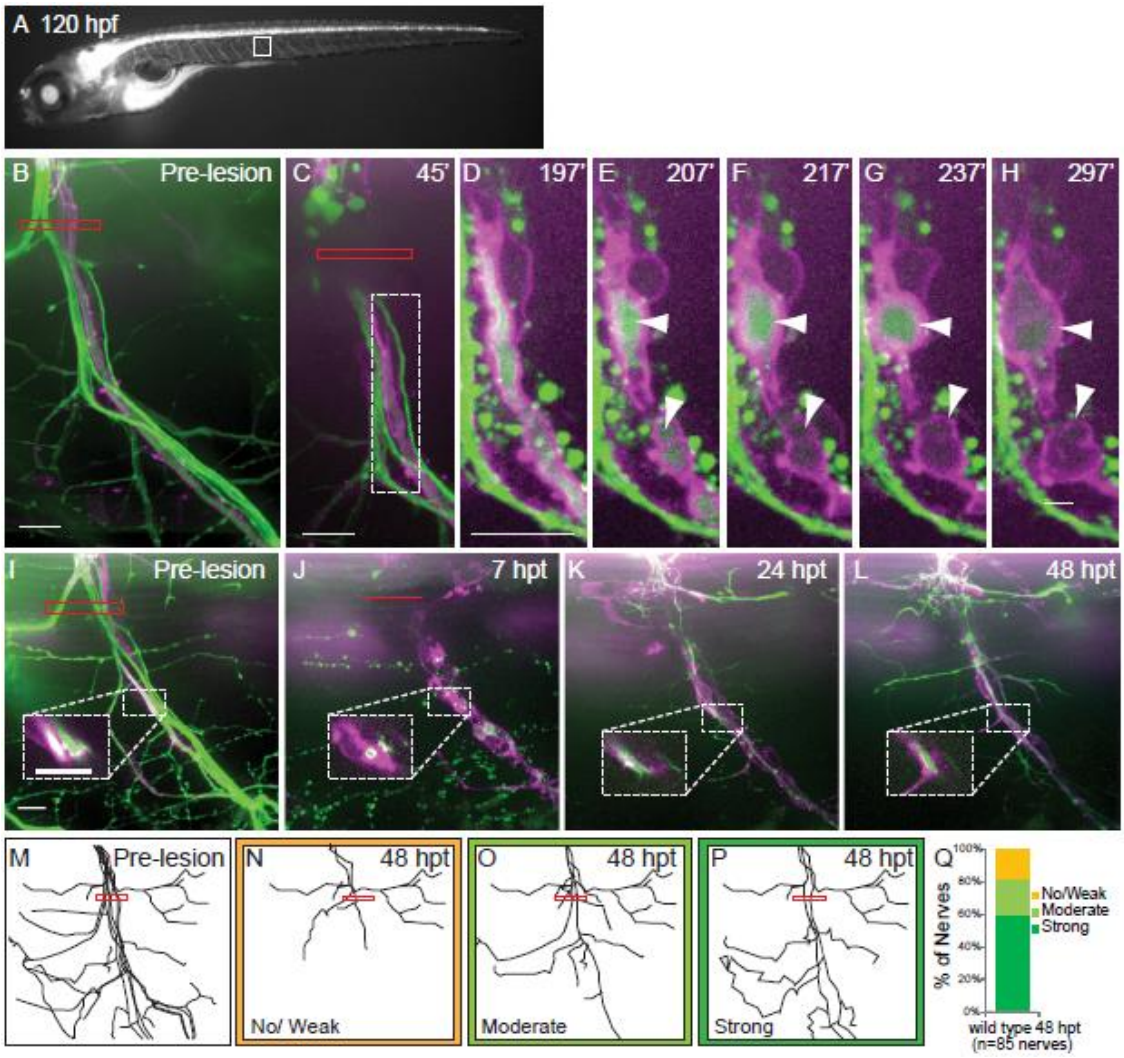


Figure 1. **Schwann cells change morphology as transected motor nerves degenerate and regenerate.** (A) 5 dpf *Tg(mnx1:GFP)* larva expresses GFP in spinal motor neurons and axons. White box: single motor nerve. (B-H) Schwann cells change morphology as axons fragment. (B, I) Pre-lesion nerve in 5 dpf *Tg(mnx1:GFP; sox10-mRFP)* larva. Red box: area of laser axotomy. (C-H) As axons fragment Schwann cell membranes (magenta) surrounding axons (green) change morphology and (D-H) in 2 hours begin degrading axonal debris contained within their membranes (arrowheads, E-H). (I-L) Schwann cell membrane morphology changes over 48 hpt (Insets: 1 µm z-plane) (J) At 7 hpt distal axons have completely fragmented and remaining denervated Schwann cell membranes appear gnarled. (K) At 24 hpt axons have regrown into the ventral myotome along original trajectory where Schwann cells remained. (L) At 48 hpt following axon regeneration Schwann cell membranes appear thinner and smoother like pre-lesion Schwann cells. Scale bar is 10 µm. (M-P) Cartoon regeneration categories for (Q) quantification of nerve regeneration at 48 hpt.

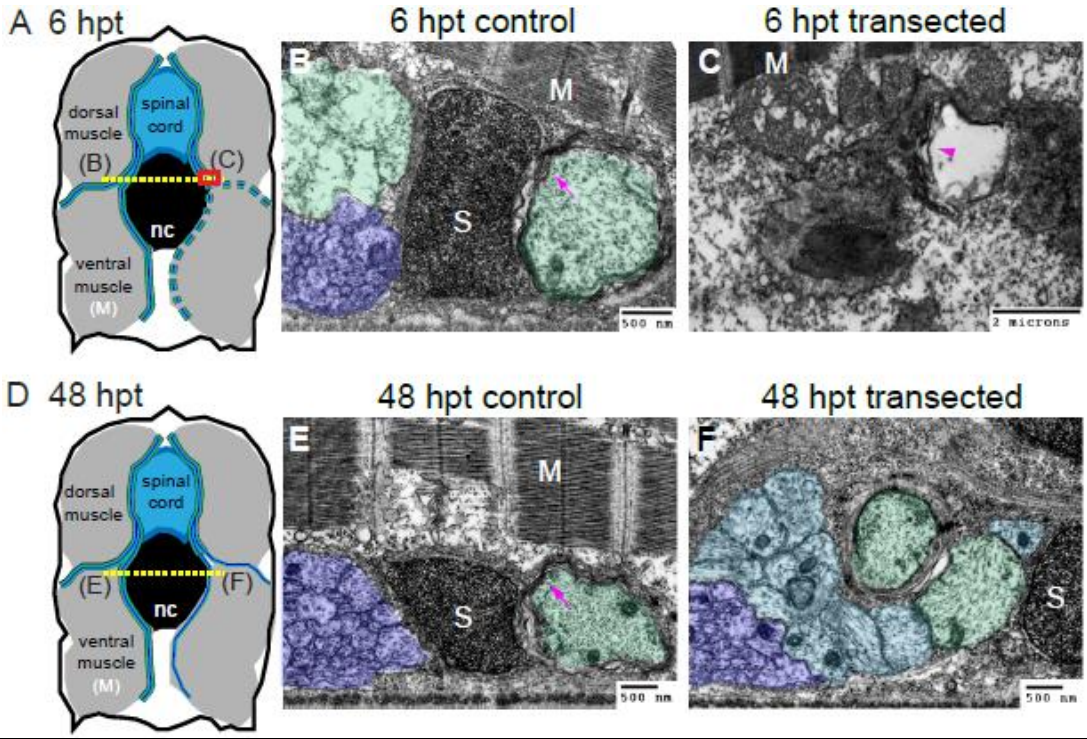


Figure 2. **Ultrastructural sections of degenerating and regenerating motor nerves.** (A, D) Cartoons roughly indicate positions of nerve slices for electron micrographs from control uncut nerves (B, E) and transected nerves (C, F) at 6 hpt (B, C) and 48 hpt (E, F). Magenta arrows: myelin; magenta arrowheads: collapsed myelin membranes; green shading: large diameter axons; teal shading: medium diameter axons; blue shading: small diameter axons; blue arrowheads: possible axon blebs; S: Schwann cell; M: Muscle.

regeneration since blocking these processes leads to misrouted axons (Cajal, 1928; Politis et al., 1982; Hall, 1986b; Stoll et al., 1989; Chen et al., 2005). To visualize Schwann cell behaviors we used larvae in which motor axons are labeled with DsRed via the *Tg(Xla.Tubb:DsRed)* transgene (Peri and Nüsslein-Volhard, 2008), and Schwann cell nuclei with the photoconvertable fluorophore Eos via the *Tg*(*sox10:nlseos)* transgene (Prendergast et al., 2012). Using an anti-sox10 antibody we first confirmed that the *sox10:nlseos* line faithfully labels all Schwann cell nuclei on ventral motor nerves (Figure 3 I-K, n=348/351 nuclei co-labeled).

We then transected and imaged motor nerves every 10 minutes from ~6 hpt through ~13 hpt, a period during which motor axons are actively regrowing (Figure 3A-G, Figure 5A-H, Movie 2 and Movie 3). Live cell imaging revealed that *sox10* positive cells migrated in ~42% of transected nerves **(**n=27 migrating cells on 19 nerves, out of 45 nerves analyzed, Figure 3H). Migration on the distal nerve stump toward the transection site was more frequent (n=24 migrating cells on 19 nerves) than migration on the proximal nerve stump toward the transection site (n=3 migrating cells on 19 nerves), suggesting that Schwann cells respond differently to nerve transection based on their relative position to the injury site. Finally, we observed dividing Schwann cells in only 14% of transected nerves (n=6 Schwann cells on 45 nerves). Given that ~ 80% of transected nerves regenerate successfully (Figure 1Q), our data suggest that Schwann cell migration and proliferation are likely less important for the initial stages of axon regeneration when axons cross the lesion site and extend towards their original trajectory.

The role of Schwann cells in motor axon regeneration

To define the *in vivo* role of Schwann cells for axonal regeneration we examined four genetic mutant strains lacking differentiated Schwann cells. Specifically, we analyzed mutants lacking the *sox10* transcription factor, which is critical for early neural crest development; mutants lacking *erbb2* or *erbb3,* which form a heterodimeric receptor critical for Schwann cell differentiation; and mutants lacking the ErbB2/ErbB3 ligand *nrg1typeIII* (Kelsh et al., 1996; Dutton et al., 2001; Lyons et al., 2005; Perlin et al., 2011). In all mutants motor axons develop without delay, and through 5 dpf their motor axons are morphologically indistinguishable from those in wild-type siblings (Figure 4A,

E, data not shown). Furthermore, the absence of Schwann cells did not affect Wallerian degeneration and debris removal (Figure 4F, G, and Rosenberg et al., 2012). Compared to wild type siblings, axon regeneration was severely affected in all mutants lacking Schwann cells (Figure 4 C, D, compared to G, H). Quantification of axon regeneration at 48 hpt revealed that in mutants lacking Schwann cells, 50-80% of transected nerves regenerating axons failed to extend along their original trajectory through the ventral myotome (Figure 4I compared to <20% in wild type siblings). Instead, regenerating axons grew extensively into lateral territories, where they formed thick fascicles through the lateral myotome in areas that are sparsely innervated both pre-lesion and following regeneration in the presence of Schwann cells (Figure 4G, H), suggesting that Schwann cells direct regenerating axons. We therefore asked whether Schwann cells influence growth rates and/or growth direction when regrowing axons cross the injury gap and extend towards their original trajectory. Time-lapse analyses of regenerating wild type axons revealed that regeneration commences as early as 3-5 hpt, when multiple growth cones sprouted from the proximal stump in all directions (data not shown). Beginning around 5-7 hpt growth cones had crossed the injury gap (Figure 5A-C, Movie 3), and then over the next few hours rapidly pioneered a path along their original trajectory (Figure 5C-E). Additional regenerating axons joined and formed fascicles with the pioneer axons, and over the next ten hours these fascicles extended through the entire length of the ventral myotome (Figure 5E-H).

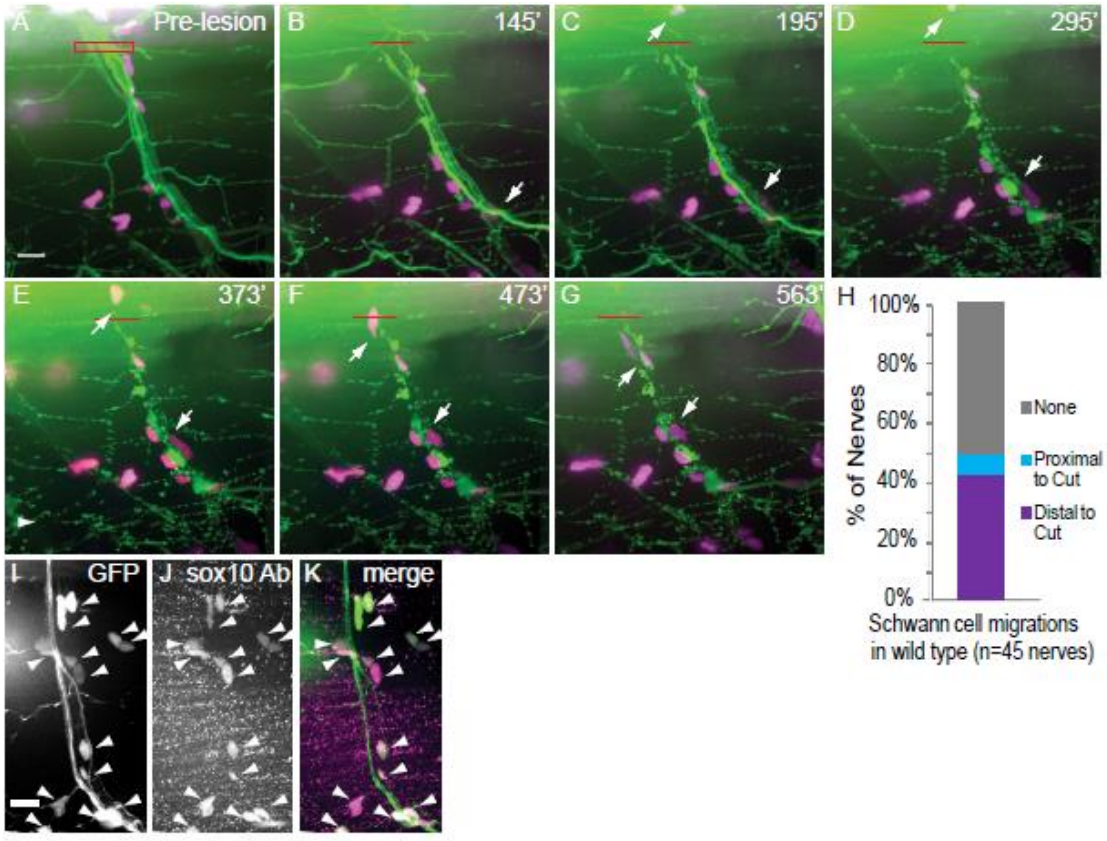


Figure 3. Schwann cells migrate following transection. (A) Pre-lesion nerve in 5 dpf *Tg(sox10:nlseos; Xla.Tubb:DsRed)* larva. Red box: area of laser axotomy. (B-G) Schwann cells labeled with nuclear GFP (pseudocolored magenta) migrate from distal and proximal locations towards the transection gap (arrows: leading edge of nucleus). The proximal Sox10+ cell crosses the transection gap (F, G). (H) Quantification of Schwann cell nuclear migration. (I-K) Schwann cells labeled in the transgenic *Tg(sox10:nlseos;mnx1-GFP)* line are Sox10+. (I) GFP labels the motor nerve and Schwann cell nuclei. (J) Sox10 antibody labels all Sox10+ nuclei along the motor nerve (K) merge of GFP (green) and Sox10 antibody staining (magenta; n=346 nuclei co-localize, 2 are only GFP+, and 5 only Sox10 antibody+). Scale bar is 10 µm.

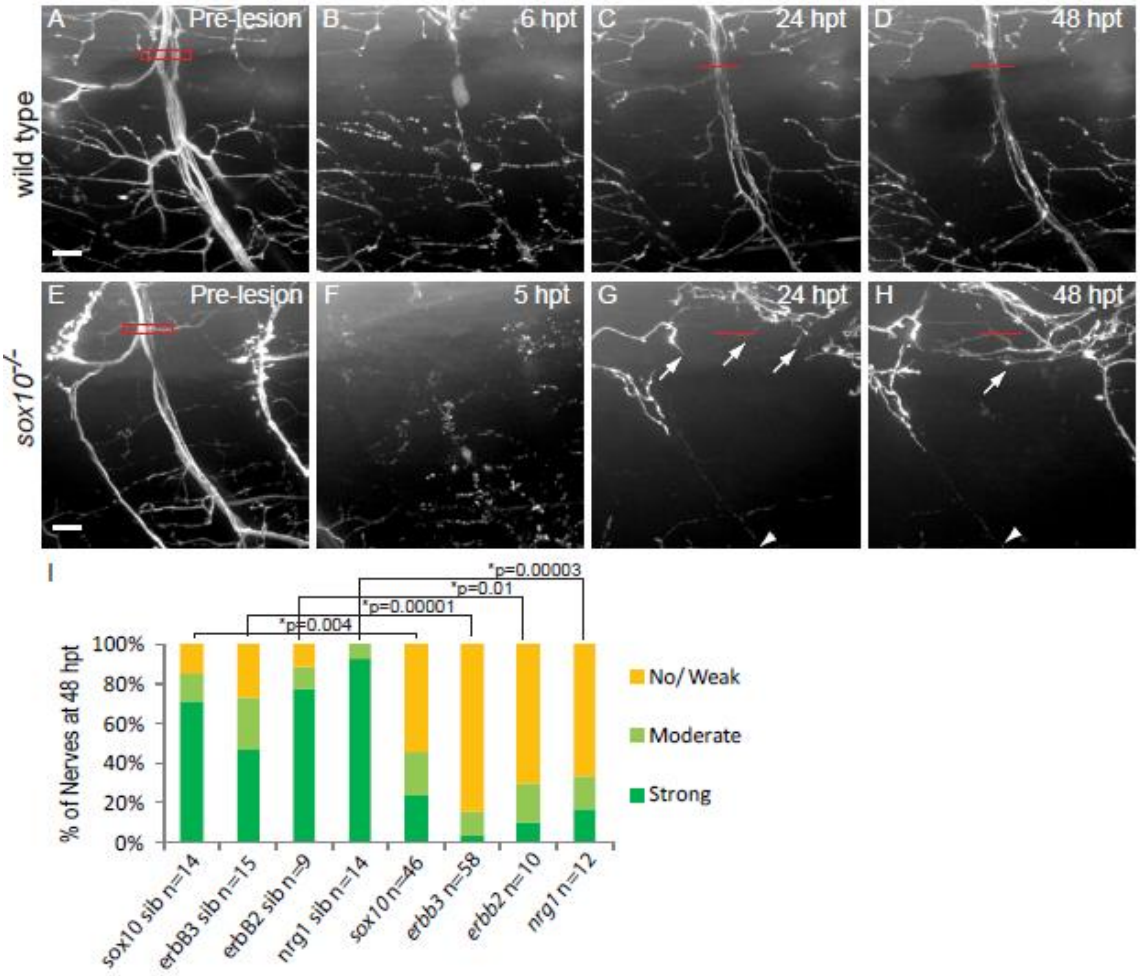


Figure 4. In the absence of Schwann cells regenerating axons extend along ectopic trajectories. (A-D) Axonal regeneration in wild-type animals. (A) Pre-lesion nerve in wild-type sibling *Tg(mnx1:GFP)*. (B) By ~6 hpt axons have completely fragmented. (C, D) At 24 hpt and through 48 hpt axons regrew into the ventral myotome, along the full extent of the original path, and branched. (E-H) Axon regrowth is misguided following transection in larvae lacking Schwann cells. (E) In a *Tg(mnx1:GFP); sox10*$^{-/-}$ mutant nerve appears normal before lesion. (F) 5 hpt nerve fragmentation and debris removal appears normal. (G, H) Axons sprout and extend from the proximal stump yet fail to grow along the original, central myotomal path and do not reinnervate the ventral half of the ventral myotome (arrows: most ventral regrowth, arrowheads: regrowth along hemisegment boundary). Red box: area of laser axotomy. Scale bar is 10 μm. (I) Quantification of the extent of nerve regrowth at 48 hpt using categories established in Figure 1 in siblings and mutants that lack Schwann cells: (*sox10*$^{-/-}$, *erbB3*$^{-/-}$, *erbB2*$^{-/-}$, and *nrgItypeIII*$^{-/-}$).

Analysis of *sox10*$^{-/-}$ mutants revealed that regenerative growth cones sprouted and extended from the proximal stump in multiple directions, indistinguishable from those in wild type (Figure 5I-P and Movie 4; n=16 nerves). Importantly, average growth rates and maximum growth rates of these misguided axons in mutants were similar to rates observed in wild type (wild type: 0.22 mm/day average with a maximum rate of 0.50 mm/day; n=8 axons from 8 nerves in 6 animals; *sox10*$^{-/-}$ mutants: 0.23 mm/day average rate with a maximum rate of 0.44 mm/day; n=10 axons from 9 nerves in 6 mutants). However, in contrast to wild type, growth cones in *sox10*$^{-/-}$ mutants continued to extend in all directions, without establishing a growth preference towards their original path (Figure 5I-P). Furthermore, axons that initially grew in the appropriate ventral direction rarely extended ventrally beyond the level of the lateral line, and instead grew along ectopic lateral trajectories (Figure 5L, P). Thus, in the absence of Schwann cells, regenerating axons retain the capacity to sprout growth cones and extend, however they stray onto multiple, aberrant trajectories, failing to stabilize towards their original developmental path. Combined, these genetic analyses provide compelling evidence that within a few hours after axons sprout from the nerve stump, Schwann cells provide regenerating growth cones with guidance critical for axons to navigate towards the original path.

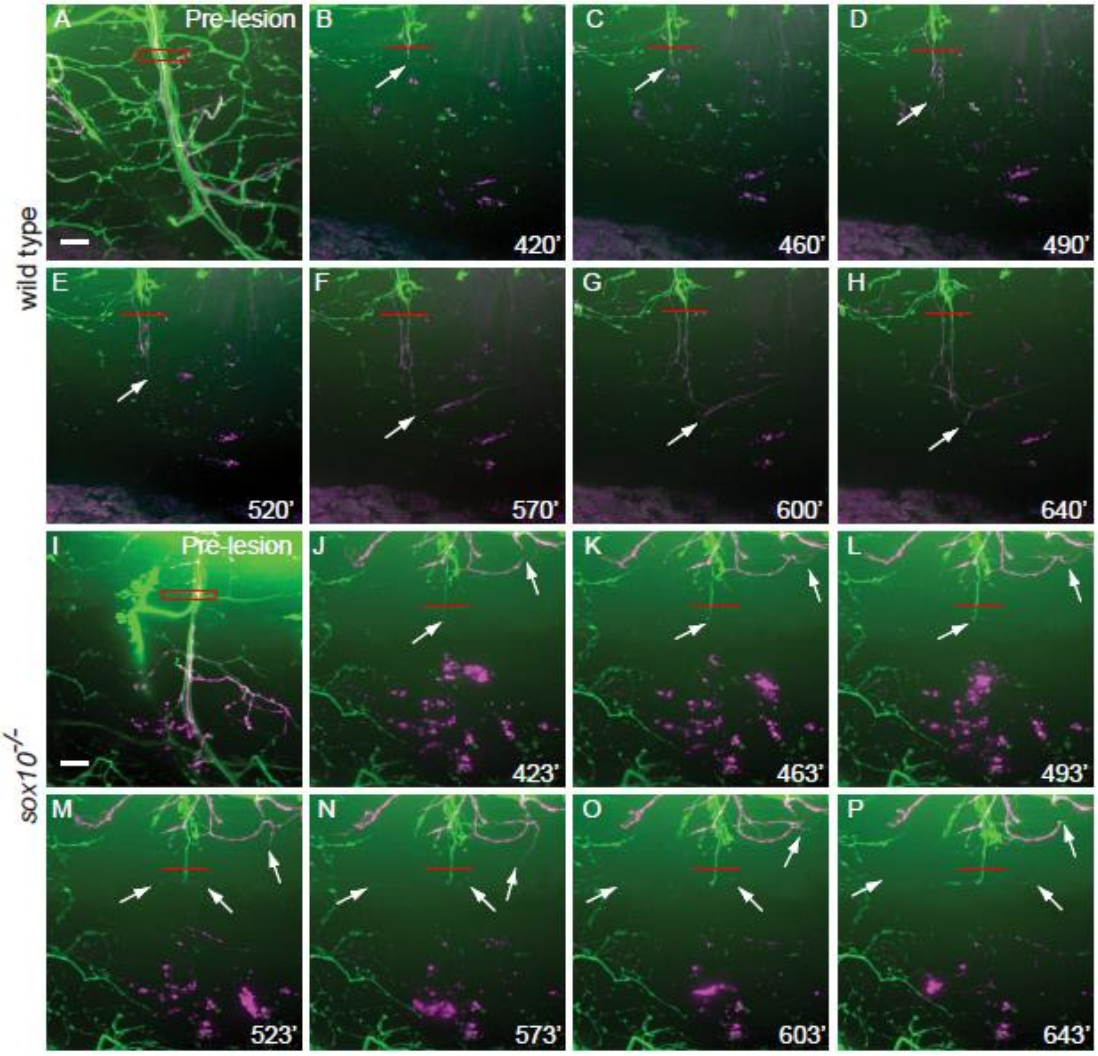


Figure 5. Schwann cells direct regrowing axons as they extend from the proximal stump. (A-H) Nerve regeneration timecourse in wild-type *Tg(mnx1:GFP)* nerves. Axons extend across the lesion gap, into the ventral myotome, and fully traverse the original axonal path over several hours. (I-P) Axons sprout and extend from the proximal stump following transection in *Tg(mnx1:GFP); sox10*$^{-/-}$ mutants, but retract frequently and do not progress along the original ventral path. Red box: area of laser axotomy, magenta: singly labeled axons, arrows: most ventral sprouts. Scale bar is 10 μm.

Axonal scaffolds fail to compensate for Schwann cell dependent guidance

Our data are consistent with the idea that as growth cones sprout from the proximal nerve stump, Schwann cells direct regrowing axons onto their original path. Conceptually this

could be accomplished through several mechanisms. The prevailing view in the literature suggests that denervated Schwann cells and their surrounding basement membrane act as a substrate, sufficient to outline a path towards their original targets (Cajal, 1928; Ide et al., 1983; Hall, 1986b; Chen et al., 2005; McDonald et al., 2006; Parrinello et al., 2010). Alternatively, denervated Schwann cells in the distal nerve stump might produce factors that direct regrowing axons onto the original path, however, the identity of such factors has remained largely elusive (Politis et al., 1982; Kuffler, 1986; Heumann et al., 1987). During development when differentiated Schwann cells are absent, axons fasciculate with and grow along other axons (Tessier-Lavigne and Goodman, 1996; McDonald et al., 2006), suggesting that axons form a suitable substrate to support axonal growth. Furthermore, our live cell imaging reveals that regenerating axons fasciculate with pioneering axons (Figure 5D-H).

To determine whether a physical scaffold delineating the original path is sufficient to guide motor axons, we generated two experimental paradigms that provide a permissive scaffold devoid of Schwann cells. First, we took advantage of a transgenic line we had previously generated, in which axonal fragmentation is delayed for over a week due to overexpression of the Wallerian Degeneration Slow (Wld^S) protein in motor neurons (Rosenberg et al., 2012). We therefore transected nerves in larvae lacking Schwann cells and expressing Wld^S selectively in motor axons (*Tg(mnx1:GFP; Wld^S-eGFP)*; *sox10$^{-/-}$*) to generate an axonal scaffold distal to the transection gap completely lacking Schwann cells (Figure 6). Consistent with previously published results (Lunn et al., 1989; Rosenberg et al., 2012), we find that in the presence of Schwann cells, regenerating axons extended alongside and intermingled with Wld^S positive axons,

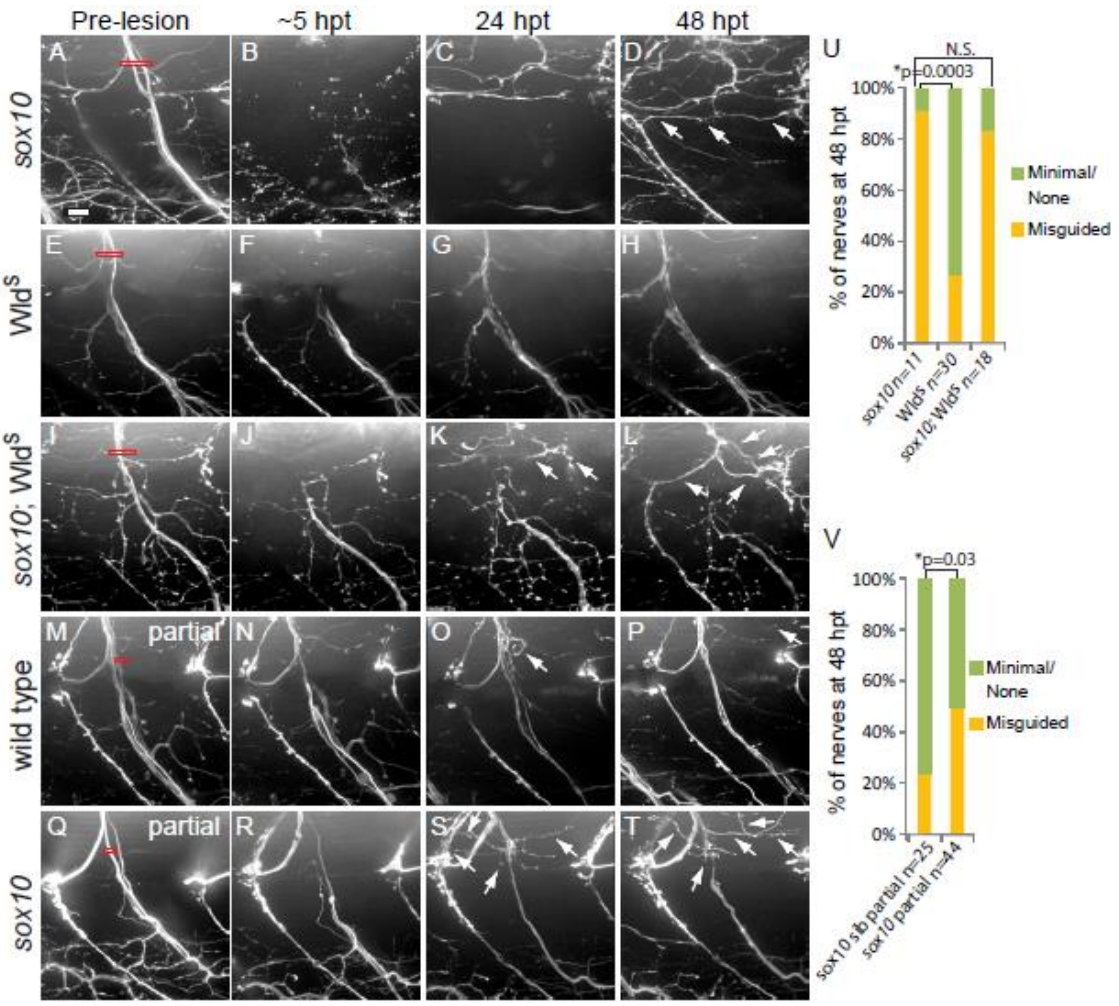


Figure 6. A physical axonal scaffold fails to compensate for the absence of Schwann cells. Red box: site of either full or partial nerve transection. (A) Pre-lesion nerve in a *Tg(mnx1:GFP); sox10*$^{-/-}$ mutant (B) 5 hpt (C, D) regrowth is misguided after 24 hpt and 48 hpt. (F-H, J-L) Axonal tissue distal to nerve lesion is Wld^{s} positive and will remain intact for a week following lesion. (E) Pre-lesion nerve in a *Tg(mnx1:GFP; mnx1:Wld*^{s}*-eGFP)* (F) 5 hpt distal nerve remains intact. (G, H) Regrowing axons fasciculate and grow along Wld^{s}+ distal nerve scaffold. (I) Pre-lesion nerve in a *Tg(mnx1:GFP; mnx1:Wld*^{s}*-eGFP); sox10*$^{-/-}$ mutant (J) 5 hpt distal nerve remains intact. (K, L) Regrowth is misguided despite Wld^{s}+ distal nerve scaffold. Following partial transection (M, Q) only axonal tissue distal to cut fascicles will fragment (N, R). (O, P) regrowth in a wild-type sibling after 24 and 48 hours post partial transection. (S, T) regrowth in *sox10*$^{-/-}$ mutant after 24 and 48 hours post partial transection is misguided despite intact axonal scaffold. Arrows: misguided regrowth. Scale bar is 10 µm. (V) Quantification of misguidance in regenerated nerves 48 hours after transection. (U) Quantification of misguidance in regenerated nerves 48 hours after partial transection.

demonstrating that they are not inhibitory to axon regrowth (Figure 6E- H). Yet despite the presence of an axonal scaffold positioned in close proximity to the proximal stump (Figure 6I-L), in larvae lacking Schwann cells regenerating axons still failed to navigate onto their original path and instead extended along ectopic trajectories identical to what we observed in *sox10*$^{-/-}$ mutants (compare Figure 6A-D, I-L, quantified in Figure 6U).

Crossing the transection gap is a well known challenge for regenerating peripheral axons (Zochodne, 2008), presenting one possible explanation why regenerating axons extended along ectopic trajectories despite the presence of an axonal scaffold along their original path. To test whether a permissive scaffold spanning the injury gap is sufficient for proper regenerative growth, we provided regenerating axons with a continuous axonal scaffold that spanned the injury gap and extended along the entirety of the original path. We generated this uninterrupted axonal scaffold devoid of Schwann cells by transecting roughly half of the 60-80 motor axons in the motor nerve (Figure 6). In wild type siblings the vast majority of regrowing axons in partially transected nerves regrew along their original trajectory, with few axons straying from appropriate trajectories at 48 hpt (Figure 6 M-P, quantified in Figure 6V). In mutants lacking Schwann cells we observed axons regenerating along ectopic trajectories in ~50% of partially transected nerves (n=22/44), down from ~90% in fully transected nerves lacking Schwann cells (Figure 5Q-T, quantified in Figure 6U, V). Thus, the presence of a continuous axonal scaffold that bridges the transection gap can only partially compensate for the role of Schwann cells in directing regrowing axons onto their original path. Combined our data provides compelling evidence that *in vivo,* Schwann cells serve regrowing axons with more than a permissive substrate that simply connects regenerating growth cones with the denervated

Schwann cells along their original path. Instead, we propose that Schwann cells actively guide axons across the injury gap and towards their original path.

The DCC guidance receptor is required for regenerative axon guidance

We next sought to define the role of canonical axon guidance systems in Schwann cell dependent guidance during regeneration. Several canonical axon guidance systems, including Netrin and its receptor Deleted in Colorectal Carcinoma (DCC) have been implicated in promoting the extent of axon regeneration (Madison et al., 2000; Tanno et al., 2005; Gabel et al., 2008; Webber et al., 2011). However, an *in vivo* function for DCC in regenerative axon guidance has not been defined. *In situ* hybridizations revealed that *dcc* mRNA is expressed throughout the spinal cord, and that *dcc* mRNA co-localizes with GFP positive motor neurons in 5 dpf *Tg(mnx1:GFP)* larvae (Figure 7J). Importantly, *dcc* mRNA in GFP positive motor neurons was maintained through the period of initial axonal regrowth at 6 hpt and 9 hpt (Figure 7K, data not shown).

To test whether DCC is required for motor axon regeneration *in vivo* we transected nerves in $dcc^{zm130198}$ mutants. The $dcc^{zm130198}$ allele carries a retroviral insertion 106 bp upstream of the start codon, resulting in a 90% reduction of *dcc* mRNA (Jain et al., 2014). Importantly, motor axons and Schwann cells develop normally in $dcc^{zm130198}$ mutants, and at 5 dpf they are morphologically indistinguishable from those in wild-type siblings (Figure 7E, data not shown). By 48 hpt we found that 78% of transected $dcc^{zm130198}$ mutant nerves extended motor axons along their original path (n=46/59 nerves), similar to what we observed in wild type siblings (90%, n=28/31 nerves). However, in about 40% of the transected nerves in $dcc^{zm130198}$, regenerating motor axons

extended not only along their original path, but also along ectopic lateral trajectories (Figure 7G, H). Importantly, misdirected axons deviated from the original path at the level of the injury gap, extending into lateral territories, and albeit less frequent, similar to what we observed in mutants lacking Schwann cells (Figure 3). Thus, *dcc* is required *in vivo* to guide regenerating motor axons across the injury gap towards their original trajectory. Combined, our results demonstrate that both Schwann cell and *dcc* dependent guidance is required during the early steps of axon regeneration to direct regenerating growth cones towards their original path.

DISCUSSION

Axons in the peripheral nervous system can re-establish functional connections with their original synaptic targets, in large part because the PNS environment promotes regrowth (Aguayo et al., 1981; David and Aguayo, 1981). Thus, to understand how peripheral axons regenerate, it is fundamentally important to visualize how injured axons interact with their cellular environment *in vivo*. We previously developed a system to record the process of motor axon degeneration *in vivo,* and to document the intricate cellular interactions between injured motor axons and macrophages (Rosenberg et al., 2012). Here, we utilize this system to examine how Schwann cells respond to nerve injury. We find that Schwann cells react to injury in synchrony with dynamic changes in axonal morphology, demonstrate that Schwann cells provide more than a simple permissive substrate to direct regenerating axons, and illustrate a role for *dcc* dependent signaling in guiding sprouting growth cones across the injury gap and towards their original path.

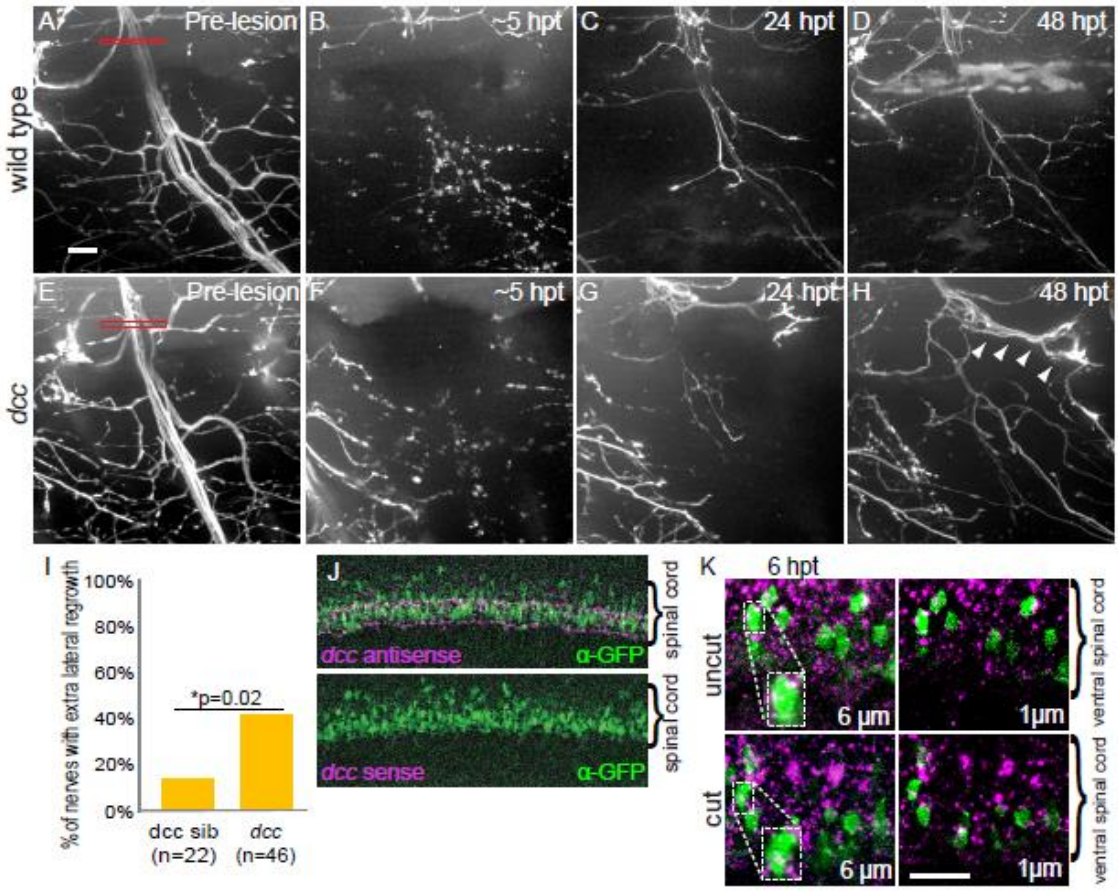


Figure 7. *DCC* directs axon regeneration. (A) Pre-lesion nerve in *Tg(mnx1:GFP)* sibling, (B) 5 hpt (C, D) and at 24 hpt and 48 hpt. (E-H) In *dcc*^{zm130198} mutant larvae, axons frequently extend on an aberrant lateral path (arrowheads). (E) Pre-lesion nerve in *Tg(mnx1:GFP); dcc*^{zm130198}mutant (F) 5 hpt (G, H). While some axons regrow along their original trajectory, many axons grow incorrectly on a lateral path (arrowheads). (I) Quantification of nerves with aberrant lateral regrowth. (J, K) In situ hybridizations for *dcc* mRNA (magenta) with antibody staining of motor neurons (green: anti-GFP) in wild type 5 dpf larvae. (J) 20X magnification of 5 dpf spinal cord with *dcc* antisense and *dcc* sense probes. (K) *dcc* mRNA levels 6 hpt on cut and uncut hemisegments from the same animal in 6 µm z-stacks and 1 µm single z-plane. Scale bar is 10 µm.

Changes in Schwann cell morphology and axonal fragmentation are highly synchronized

Schwann cells are the principle glia of the peripheral nervous system and are known to promote axonal regeneration. Following injury, Schwann cells collaborate with macrophages to degrade axon and myelin debris, clearing the path for axon regeneration. They also undergo dramatic morphological changes, reflecting their de-differentiation to a more immature state, critical for their own proliferation and production of diffusible factors that promote axon outgrowth (Holtzman and Novikoff, 1965; Scherer et al., 1984; Jessen and Mirsky, 1999; Jessen and Mirsky, 2008). However, whether axonal degeneration is synchronized with the various changes in Schwann cell behavior has not been examined in live intact animals. We find that as axons fragment, distal Schwann cells change their morphology, and over the next 24 hours retain this rounder appearance (Figure 1C-H). During the same time period Schwann cells also become motile, extending towards and sometimes across the lesion site (Figure 3). The functional significance of this early migration is unclear, as ~80% of transected nerves regenerated while Schwann cell migration was detectable in only 42% of these nerves.

Earlier work suggested that Schwann cell proliferation is important for nerve regeneration, however the time period during which proliferation is critical for regeneration has remained elusive (Hall, 1986b; Scaravilli et al., 1986). We find that within the first 10-13 hpt Schwann cell proliferation was only evident in 14% of transected nerves, consistent with the idea that Schwann cell proliferation is less important when axons cross the injury site and extend towards their original trajectory, but might become important later to replace lost Schwann cells and re-myelinate regrown axons. Finally, once axons had crossed the injury gap and extended over the denervated Schwann cells along the original path, these distal Schwann cell membranes began to revert to a smoother, pre-lesion appearance (Figure 1I-L). Thus, following nerve injury axon and Schwann cell behaviors change dramatically, and with remarkable synchronicity.

Schwann cells direct regenerating axons

Peripheral axons often regenerate successfully following crush injuries, as the basal lamina remains largely intact at the injury site, and is continuous with the band of Bungner which can serve as a substrate (Cajal, 1928; Scherer et al., 1984; Nguyen et al., 2002). In contrast, complete nerve transection disrupts Schwann cells and the basal lamina, resulting in a gap at the injury site. While peripheral axons can regenerate across the transection gap, removal of the distal stump or increasing the size of the gap diminishes the ability of axons to regenerate, indicating that the distal nerve provides critical signals that support regeneration (Lundborg et al., 1981; Lundborg et al., 1982; Lundborg et al., 1986). One perspective that dominates the literature is that Schwann cells produce neurotrophic factors that promote axon regrowth (reviewed in: Vargas and Barres, 2007).

We assessed regeneration in four mutants that lack Schwann cells without affecting development or morphology of motor axons (Figure 4), nor Wallerian degeneration (Rosenberg et al., 2012). Our time lapse movies demonstrate that early in regeneration growth cones sprout from *sox10*$^{-/-}$ nerve stumps with the same latency, and extend at similar growth rates compared to wild type, but along ectopic routes ignoring their original trajectory (Figure 4). Although we only analyzed axonal growth rates until 13 hpt, our data suggests that early during the regeneration process pioneering motor axons can rely on cell types other than Schwann cells as their source of growth promoting factors. For example, neurotrophins are known to also be expressed by skeletal muscle (Griesbeck et al., 1995), and in denervated gastrocnemius muscle, BDNF mRNA levels increase (Funakoshi et al., 1993). At the same time our results also provide compelling *in vivo* evidence that Schwann cells provide directionality to axons as they cross the injury site and navigate onto their original trajectory.

The prevailing view in the literature is that denervated Schwann cells themselves, or in conjunction with other cells such as fibroblasts, form a bridge across the lesion site and this provides regenerating axons with a permissive substrate that leads to the denervated Schwann cells in the distal nerve stump along the original trajectory (Cajal, 1928; Ide et al., 1983; Hall, 1986b, a; Chen et al., 2005; McDonald et al., 2006; Parrinello et al.,

2010). While even basal lamina extracted from peripheral nerve segments and then implanted as a scaffold at the proximal nerve is able to provide an effective pathway for regrowing axons (Ide et al., 1983), if and to which extent purely axonal scaffolds can serve as a regenerative substrate *in vivo* is unclear. We find that in the presence of Schwann cells, a continuous physical bridge consisting of non-transected axons was permissive to regrowth. In the absence of Schwann cells, such axonal scaffolds improved directional regrowth by 50% compared to when no bridge was provided (Figure 6Q-T, V compared to 6A-D, U), but failed to fully compensate for the absence of Schwann cells, and resulted in twice the frequency of misguided axons (~50%) compared to partially transected wild type nerves (~20%; Figure 6M-P, U). Thus, *in vivo*, permissive Schwann cell free substrates support partial axonal regeneration, yet fail to compensate for the robust guidance provided by Schwann cells. Importantly, our experiments do not distinguish between a contact dependent role for Schwann cells in directing regenerating axons, e.g. by providing a permissive substrate unique to Schwann cells, and a contact independent role for Schwann cells, e.g. in providing directional guidance cues emanating from Schwann cells distal to the lesion site.

DCC directs axons early during regeneration

Complete nerve transection resulting in an injury gap resembles development, when pioneering axons navigate without a physical substrate through a constantly changing environment. Expression of the canonical guidance molecules Netrin1 and Slit2 are upregulated in the distal nerve stump following transection (Wang et al., 1999; Madison et al., 2000; Tanno et al., 2005; Park et al., 2007), as are NGF and GDNF, which can act as neurotropic cues (Cajal, 1928; Letourneau, 1978; Gundersen and Barrett, 1979; Schuster et al., 2010; Arthur-Farraj et al., 2012). Guidance cues might direct proximal Schwann cells to bridge the gap first to provide a substrate for regenerating axons, or might interact directly with axonal guidance receptors. During development, DCC is expressed on axonal growth cones and responds to Netrin secreted from midline glia cells (Kennedy et al., 1994; Chan et al., 1996b; Kolodziej et al., 1996; Mitchell et al., 1996; Lauderdale et al., 1998). Following sciatic nerve transection Netrin-1 mRNA levels are

upregulated in the band of Bungner Schwann cells (Madison et al., 2000), and regenerating motor axons as well as Schwann cells proximal to the lesion site express DCC (Madison et al., 2000; Webber et al., 2011). Furthermore, siRNA knockdown of *dcc* reduces the number and extent of regenerating axon fibers (Webber et al., 2011), and in netrin-1 heterozygous mice the extent of peripheral nerve regeneration is significantly reduced (Jaminet et al., 2013). However, if and to what extent Netrin/DCC signaling guides regenerating axons has not been examined *in vivo*.

We find that prior to and in the hours following nerve transection *dcc* mRNA is detectable in motor neurons, although *dcc* expression was widespread throughout the spinal cord, consistent with *dcc*'s well documented expression (Chan et al., 1996a; Kolodziej et al., 1996. (Figure 7). Motor axons in $dcc^{zm130198}$mutants regenerate, yet a significant portion of motor axons extended along ectopic trajectories, somewhat similar to what we observed in animals lacking Schwann cells.

Future experiments will determine whether in zebrafish *dcc* function is required in regenerating motor axons to respond to Netrin, possibly secreted from distal denervated Schwann cells, to attract axons across the injury site. Alternatively, *dcc* might function in proximal nerve stump Schwann cells to respond to Netrin secreted from distal denervated Schwann cells to attract proximal Schwann cells across the injury site, thereby forming a physical bridge that regenerating axons utilize to cross the injury gap. Our combined results reveal a novel and definitive role for Schwann cells and *dcc* mediated guidance early in peripheral nerve regeneration to direct growth cones across the transection gap and onto their original axonal trajectory.

Chapter 4: Discussion

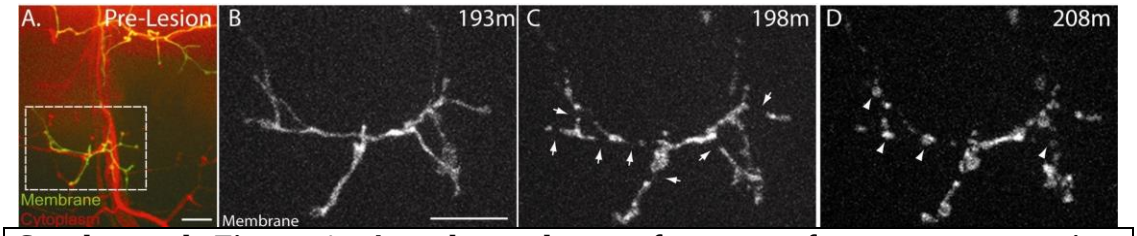
Wallerian degeneration is an early step towards functional nerve regeneration, and involves extensive cellular interactions between injured axons and multiple non-neuronal cells such as immune and glial cells. Early studies established that the environment generated by macrophages and Schwann cells is critical for successful nerve regeneration (Aguayo et al., 1981; David and Aguayo, 1981), and endpoint analysis of stained sections documented complex histological changes in Schwann cell appearance, myelin breakdown and macrophage influx following peripheral nerve injury (Martini et al., 2008). While possible, albeit labor intensive, *in vivo* imaging of nerve degeneration in murine models has been established, yet the focus has been primarily on changes in axons (Beirowski et al., 2004; Kerschensteiner et al., 2005). Thus, despite its importance, a minute-by-minute account of the cellular interactions between injured nerves and non-neuronal cells was lacking. Moreover, which of these cellular interactions are of functional significance is largely unknown. Our results characterize, for the first time and with unprecedented temporal and spatial resolution, the cellular interactions between injured nerves, Schwann cells, and immune cells. Importantly, these observations combined with genetic manipulations and cell type ablation lead to several new insights into the process of Wallerian degeneration and axon regeneration.

We have taken advantage of the transparency of the zebrafish to precisely transect mature motor nerves and to image axonal destruction, axon regeneration, and the cellular response of macrophages, perineural glia, and Schwann cells *in vivo* and in real time. By

laser transecting motor nerves we model the physical trauma that nerves undergo. By controlling the location of the laser pulse and power levels we can minimize damage to the animal and thus precisely and reproducibly sever spinal motor nerves. However, as with most physical nerve injuries, our injury also causes collateral damage. 4-5 hours after transection muscle damage is evident in brightfield images, appearing puckered over nerve lesion sites. TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling) is a method for detecting DNA fragmentation, and thus apoptotic cells, by labeling the terminal end of nucleic acids. We also see TUNEL positive muscle cells at the site of laser ablation after 4-5 hours (data not shown). This damage is evident in electron micrographs, which show widespread damage throughout the fast-twitch muscles that overlay the transected nerves (Chapter 3 Figure 1 w, x). This damage is still apparent 48 hours after injury via electron micrograph. (Chapter 3 Figure 1 y, damaged muscle between intact muscle and basement membrane). Thus, we conclude that our nerve transections, while precise and reproducible, still cause collateral damage much as any other traumatic injury would. This should activate a host of inflammatory signaling that is thought to be essential to the regenerative process (Lu and Richardson, 1991; Perry and Brown, 1992b).

Overall, we find that nerve degeneration proceeds with the same morphological landmarks as those reported for Wallerian degeneration in mammals, and we also confirmed axon fragmentation in using an axonal membrane tag (Chapter 1 Figure 1, Supplemental Figure 1) (Adalbert et al., 2005b; Beirowski et al., 2005; George et al., 1995; Lunn et al., 1989a; Martin et al., 2010; O'Daly and Imaeda, 1967; Raff et al., 2002; Vargas and Barres, 2007; Waller, 1849). The lag time between injury and onset of axonal

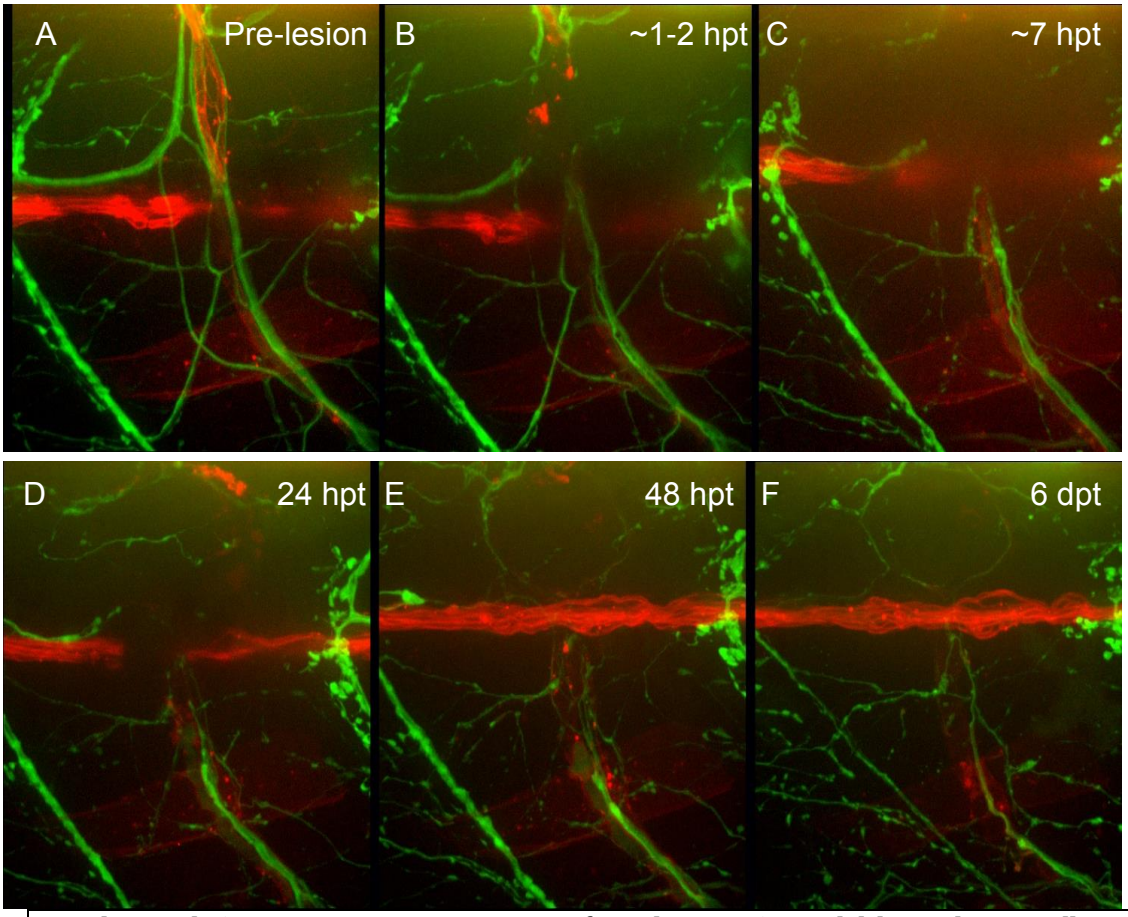
degeneration is known to vary significantly between vertebrate species (Vargas and Barres, 2007), and importantly, the lag time of 121-240 minutes we observed in zebrafish remained constant as larvae aged (6-14 dpf; data not shown), consistent with previous observations that motor nerves at 5 dpf have established mature trajectories and connections (Westerfield and Eisen, 1988b). Moreover, we find that expression of Wld^{s} in motor nerves effectively delays fragmentation, as previously reported in zebrafish sensory axons and in other species (Adalbert et al., 2005a; Adalbert et al., 2005b; Araki et al., 2004a; Araki et al., 2004b; Hoopfer et al., 2006a; Lunn et al., 1989b; MacDonald et al., 2006a, b; Martin et al., 2010; Raff et al., 2002; Wang et al., 2001a; Wang et al., 2001b)These data validate the zebrafish as a model for axon degeneration.



Supplemental Figure 1. Axonal membranes fragment after nerve transection. Tg(NBT:dsRed; Mnx1:mCD8-GFP). Scale bar is 10 um.

As Nmnat1 is thought by certain groups to be the main functional component of the Wld^{s} protein, we also generated a Nmnat1 transgenic line, Tg(Mnx1:Nmnat1-GFP), (Araki et al., 2004a; Babetto et al., 2010; Sasaki et al., 2009). This line, like the Tg(Mnx1:Wld^{s}-GFP) line, also has nuclear GFP and protects axons from fragmentation for about a week (Supplemental Figure 2). This fragmentation delay is equal to the protection seen in our Wld^{s}-GFP line, however, we cannot conclude that these two proteins offer equal protection from fragmentation, as Wld^{s}'s protection is dose

dependent (Mack et al., 2001), and we did not determine the levels of protein produced in each transgenic lines.



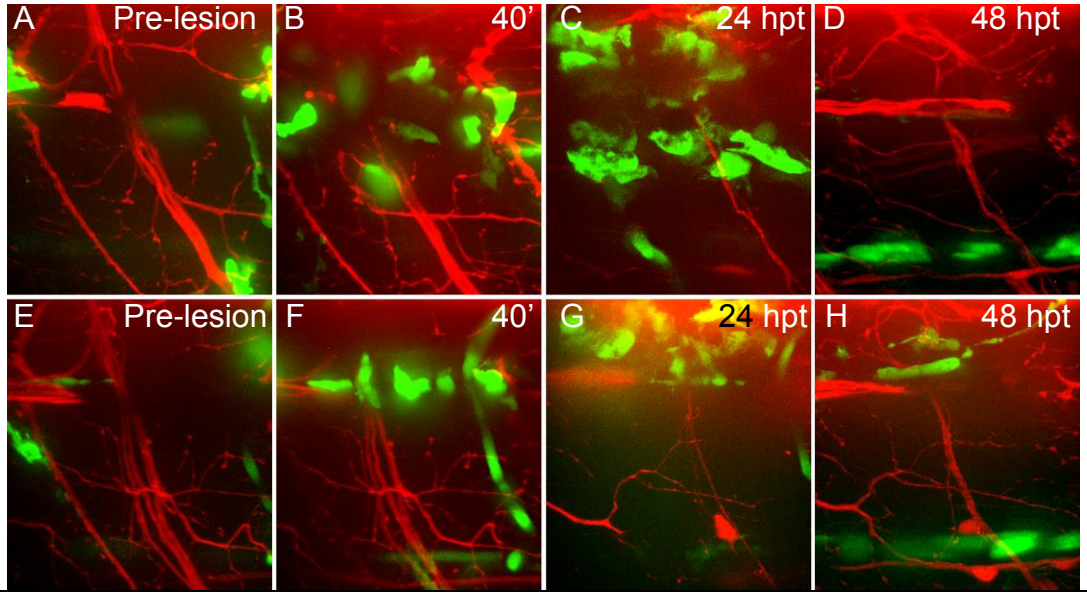
Supplemental Figure 2. Nmnat1 protects axons from degeneration and delays Schwann cell morphology changes. Following transection axons in the Tg(Mnx1:GFP; Mnx1:Nmnat1-GFP; Sox10:mRFP) line remain intact for days beyond the usual fragmentation window, which closes at ~260 minutes post transection. Schwann cell morphology also does not change until after axons fragment.

Many neonatal and young motor neurons die after axonal injury in mouse and rat (Koliatsos et al., 1993; Snider and Thanedar, 1989). We demonstrate that motor neurons survive transection in 5 dpf larvae. We proved this by individually labeling cell bodies, transecting nerves and imaging these cell bodies over the following 48 hours (Chapter1 Figure 4 E, F, G), and by TUNEL staining for apoptotic nuclei at 30 minutes, 1, 2, 3, 4,

and 7 hpt. TUNEL staining at all time points revealed that the vast majority of neurons survived transection, equal to neuron death in uncut segments (data not shown). Alternatively, it is also possible that the TUNEL staining was performed too early to see apoptotic cells. Our survival rates suggest that 5 dpf zebrafish motor neurons are roughly as mature as 3 week old rat neurons (Acheson et al., 1995; Kuno, 1990; Zochodne, 2008).

Our live cell imaging demonstrates that macrophages arrive at the lesion site long before the onset of fragmentation, independent of Schwann cells. Early studies, based on fixed samples, reported that 24 hours following sciatic nerve crush macrophage counts were not increased, and that macrophages arrived at the lesion site by the second day, when nerve fragmentation is well underway (Avellino et al., 1995; Monaco et al., 1992; Perry et al., 1987; Stoll et al., 1989a; Stoll et al., 1989b); (Bruck, 1997; George and Griffin, 1994a, b). Recent studies show that at 36 hours post lesion sciatic nerve fragmentation is accompanied by macrophages, yet the precise arrival of macrophages in relation to nerve fragmentation had not been established (Beirowski et al., 2004). We find that macrophages arrive at the lesion site 60-120 minutes prior to the onset of axonal fragmentation, independent of Schwann cells (Chapter 2 Figure 5, Figure 7). Following axonal fragmentation, macrophages infiltrate the distal portion of the injured nerve and phagocytose debris, as previously observed in mammalian systems (Macrophage activities are discussed more fully in Chapter 2, and all statistics are listed in Supplemental Table 1) (Perry et al., 1987; Stoll et al., 1989b). We also see that macrophages remain at injured nerves for days after injury, and specifically at the nerve lesion site and not at the distal nerve, suggesting a role for macrophages beyond debris removal. Macrophages may remain to provide neurotrophic factors through regeneration,

(Supplemental Figure 3) (Arthur-Farraj et al., 2012; Brown et al., 1991; Jessen and Mirsky, 2008; Zochodne, 2012); alternatively, macrophages may remain to manage the extensive tissue damage induced by laser, which is still apparent days later (Chapter 3 Figure 1Y).



Supplemental Figure 3. Macrophages remain at injured nerves after axonal debris is removed in wild type larvae (Tg(NBT:dsRed; Pu.1-GAL4-UAS-GFP)). (A-D) Macrophages persist at regenerating nerve through 24 hpt. (E-H) Macrophages persist at regenerating nerve through 48 hpt.

Interestingly, our studies uncover a previously unknown behavior of macrophages when confronted with injured Wld^{S} nerves in which fragmentation is delayed. In the first ~10 hours following transection macrophages maintain extensive contact with the Wld^{S} expressing nerve stump, and exhibited a novel 'scanning' behavior, repeatedly extending and retracting a process along the distal nerve (Chapter 1 Figure 8). This behavior was distinct from macrophage behavior following wild-type axon fragmentation. While our observations are consistent with the idea that the prolonged presence of macrophages is

pU.1 leukocyte (MΦ) statistics	Wild Type	Wld^{s} Positive	*sox10*$^{-/-}$
Average MΦ infiltration time (mins post lesion)*^	**66.7**	35.3	47.1
Median MΦ infiltration time^	41	33.5	25
Maximum MΦ infiltration time (if macs ever seen invading)	310	90	205
Minimum non-zero MΦ infiltration time^	8	4	12
Average # of MΦ in hemisegment before fragmentation, after lesion	2	4	2
Median # of MΦ in hemisegment before fragmentation, after lesion	2	4	3
Maximum # of MΦ in hemisegment before fragmentation, after lesion	8	6	4
Minimum # of MΦ in hemisegment before fragmentation, after lesion	0	0	0
Average # of MΦ in hemisegment after fragmentation	3	3	3
Median # of MΦ in hemisegment after fragmentation	3	4	4
Maximum # of MΦ in hemisegment after fragmentation	8	6	5
Minimum # of MΦ in hemisegment after fragmentation	0	0	0
# of hemisegments analyzed for MΦ counts (rows 4-12)	36	14	7
*N.B.: times would be higher if we took "never saw MΦ " into account.			
^times would be lower, but there is a delay after transection and imaging			

Supplemental Table 1. numbers and timing of macrophage recruitment to peripheral nerves following transection in 5 dpf Wild Type (*Tg(X1a.Tubb:DsRed; spi1-Gal4,UAS-GFP)*), Wld^{s}+ (*Tg(X1a.Tubb:DsRed; spi1-Gal4,UAS-GFP; mnx1:Wld^{s}-GFP;)*, and *Sox10*$^{-/-}$ *(Tg(X1a.Tubb:DsRed;spi1-Gal4,UAS-GFP*; *sox10*$^{-/-}$) larvae.

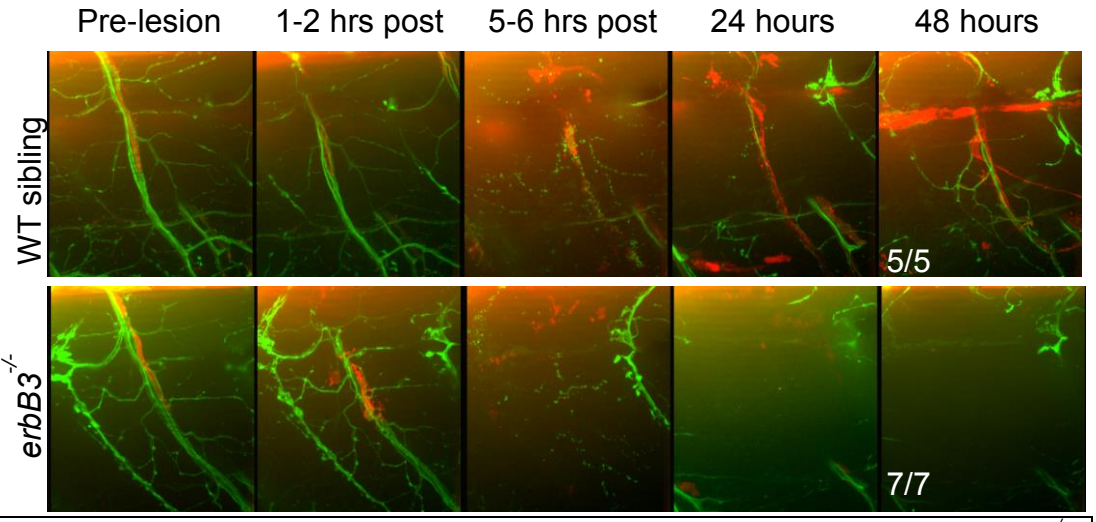
insufficient to trigger axonal fragmentation, they also suggest that macrophage behavior is modulated by nerve integrity. While the absence of axonal fragmentation does not abrogate macrophage recruitment to an injured nerve, it alters 'on site' macrophage behavior. This is somewhat reminiscent of axonal injury in the CNS, where resident microglia migrate with some delay to the lesion site, but for unknown reasons exert limited phagocytic activity (Barron, 1995; Cui et al., 2009; Lawson et al., 1994). This low rate of phagocytic activity contributes to the slower Wallerian degeneration rate in the CNS, compared to the PNS, although the rates of axonal fragmentation in the PNS and the CNS are comparable (George and Griffin, 1994a; Stoll et al., 1989a), and thus cannot

account for the difference in phagocytic behavior. In contrast, the ‘scanning’ behavior we observe is only exhibited in the absence of nerve fragmentation. Such behavioral plasticity exhibited by leukocytes has previously not been reported and strongly suggests the existence of multiple, possibly independent signals regulating macrophage activity. A first signal attracts leukocytes to the injured nerve, while subsequent signal(s) initiate leukocyte invasion and phagocytosis of the distal nerve. Future studies are required to identify these signals.

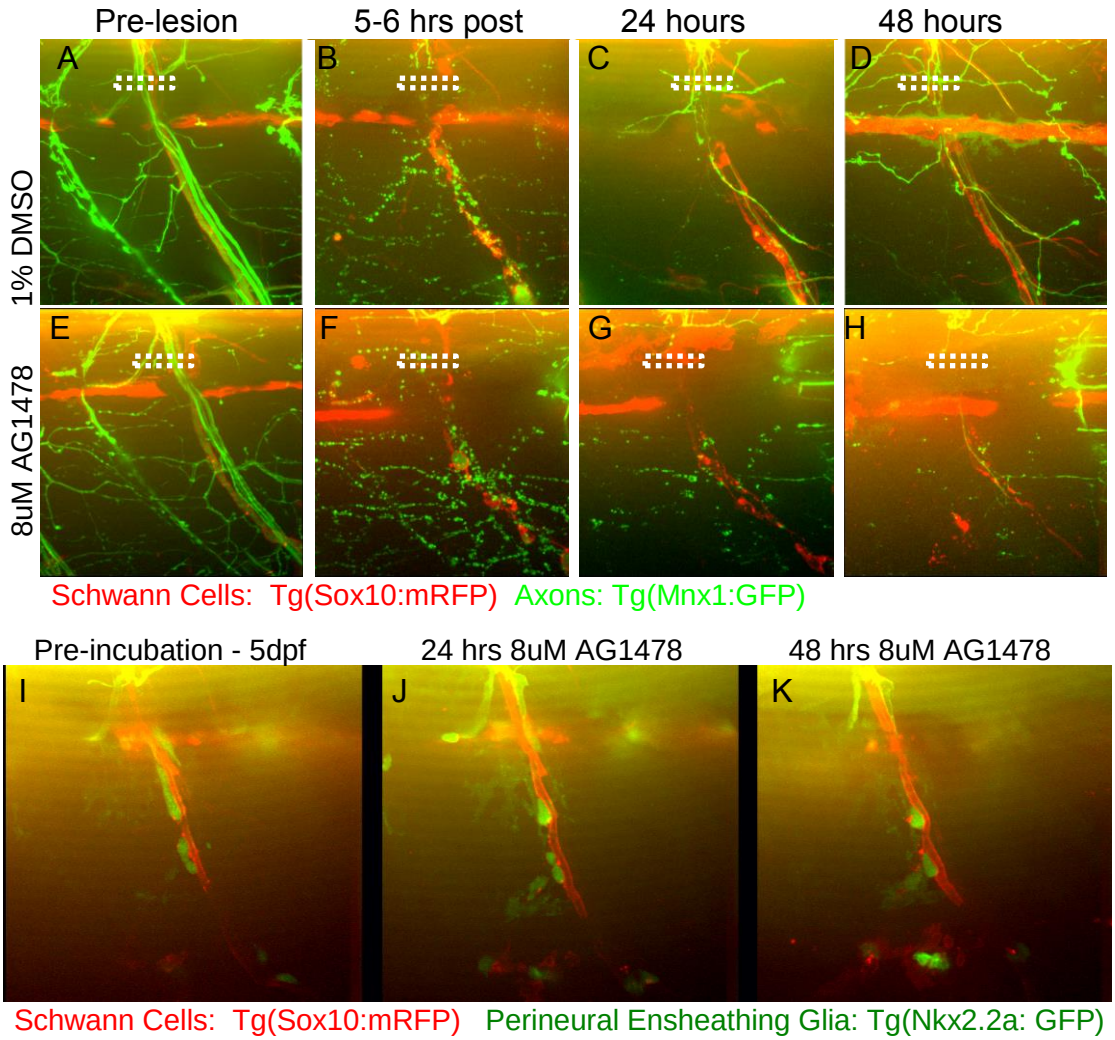
Following axon fragmentation, Schwann cells are known to change morphology and alongside macrophages degrade axonal debris (Holtzman and Novikoff, 1965; Jessen and Mirsky, 1999; Scherer et al., 1984). We see these same morphology changes, but demonstrate that they occur with remarkable synchronicity to axon fragmentation (Chapter 2 Figure 1E-H). This appears likely to accommodate the changing shape of axons as they fragment into spherical blebs. Additionally, Schwann cell membranes remained static after nerve injury in the Tg(Mnx1:Nmnat1-GFP; Mnx1:GFP; Sox10:mRFP) line (Supplemental Figure 2), where like the Wld^{s} transgenic line the distal nerve remains intact for about a week after transection. This suggests that axon fragmentation itself may signal to Schwann cells that nerves have sustained an injury, and activates Schwann cells to commence regenerative roles. This is consistent with prior literature indicating that delaying axon degeneration prevents the progression of glial events (George and Griffin, 1994b; Lunn et al., 1989a).

What molecules signal to Schwann cells that axons have sustained an injury remains unclear. It is posited that changes in axonal neuregulinI typeIII, which can bind to Schwann cell ErbB2/ErbB3 receptors may transmit this signal (Guertin et al., 2005;

Vargas and Barres, 2007). Based on this theory we attempted to block injury signaling and activation of Schwann cells after injury. As Schwann cells die after transection in *erbb3*$^{-/-}$ mutants (Supplemental Figure 4), we utilized a pharmacological agent, AG1478, known to block Nrg1/ErbB2/ErbB3 signaling (Lyons et al., 2005). Application of this agent reduced axon regeneration wholesale, an effect much stronger than when Schwann cells are genetically ablated (Supplemental Figure 5, compared to Chapter 3 Figure 3). This suggested that this agent has pleiotropic effects. We are seeking alternative ways to block Nrg1/ErbB2/ErbB3 signaling, possibly through a heat-shock rescue construct expressed in *erbb3*$^{-/-}$ mutants, activated during development to rescue Schwann cell migration and differentiation, and then left off during regeneration to block this signaling cascade. As ErbB3 signaling feeds into a PKA/AKT survival signaling cascade, these studies may have to be executed in a *p53*$^{-/-}$ background to try to keep the Schwann cells alive.



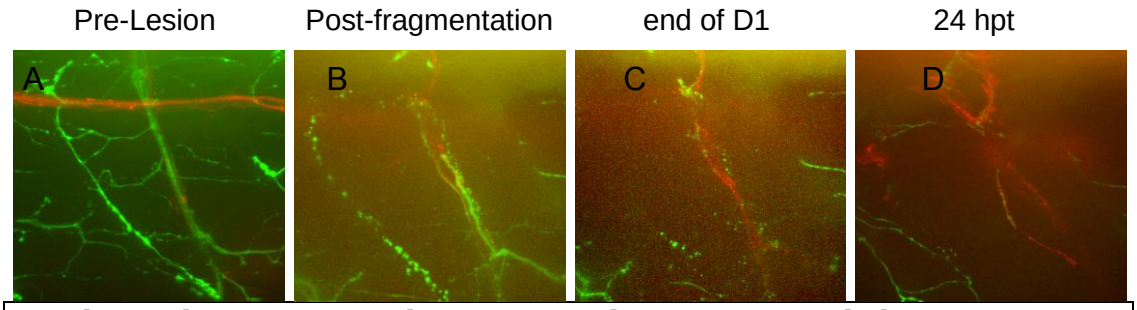
Supplemental Figure 4. Distal undifferentiated neural crest cells die in injured *erbB3*$^{-/-}$ mutants following nerve transection.



Supplemental Figure 5. AG1478 treatment reduces the overall number of regenerating axons compared to DMSO control group (A-D compared to E-H). Two days of AG1478 treatment does not affect existing Schwann cells or perineural glia in uninjured animals.

Schwann cells and macrophages are known to collaborate to remove axon and myelin debris (Holtzman and Novikoff, 1965; Jessen and Mirsky, 1999; Perry et al., 1987; Scherer et al., 1984; Stoll et al., 1989b). We demonstrate that Schwann cells are not necessary for debris clearance following fragmentation in *sox10*$^{-/-}$, suggesting that macrophages can perform this role solo (Chapter 3 Figure 3 F, G). It also appears that debris can be mostly cleared in the absence of macrophages. In *cloche*, a yet unmapped

line that completely lacks all vascular, erythroid, and myeloid lineages (Stainier et al., 1995), Schwann cells clear debris along the main trunk, where Schwann cells marked by Sox10:mRFP are evident. However, where Sox10:mRFP, and thus Schwann cells, are absent along branches further from the main trunk it appears as though debris is cleared to some extent (Supplemental Figure 6). The *cloche* larvae are very sick and necrotic due to an overall lack of circulation and oxygen to their tissues, which it turn makes muscles translucent and difficult to image through, and I hesitate to draw conclusions from these studies. It is possible that the fluorescence of the debris fades faster in these animals, or that it is harder to see the debris due to the difficulty in imaging. Alternatively, it is possible that the debris is removed by a third cell type. In zebrafish sensory axons the Sagasti lab reported that a third cell type removes debris, in their case, thought to be the skin itself (Martin et al., 2010). We sever motor nerves close to the notochord, which far from epidermal layers, indicating that there may be addition non-skin, non-myeloid, and non-glial cells involved in debris removal.

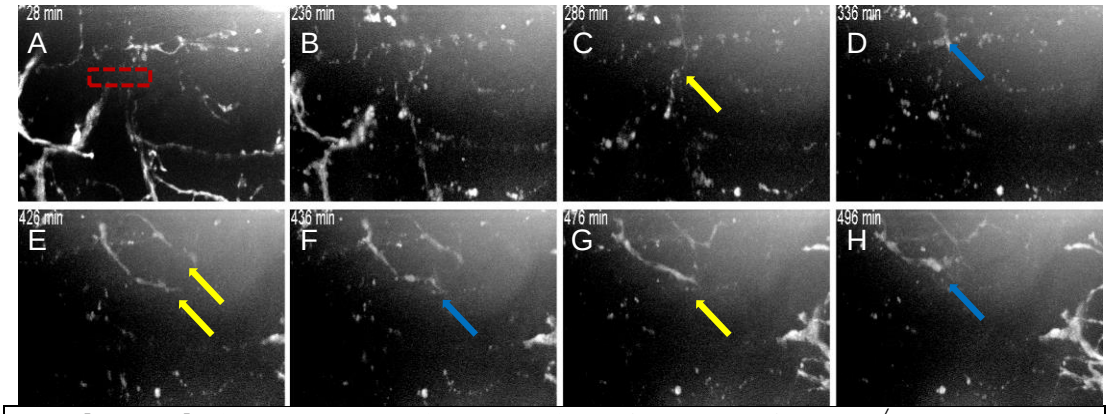


Supplemental Figure 6. Axon degeneration and regeneration in *cloche* mutants. Nerves fragment normally (B), and debris is removed along the nerve trunk as well as distal regions (C), and a few axons regenerate by 24 hpt (D). Tg(Mnx1:GFP; Sox10:mRFP); *cloche*

Does debris matter in the peripheral nervous system? In the CNS, myelin debris is inhibitory to regrowing axons. PNS myelin debris contains molecules like MAG that are

traditionally inhibitory to outgrowth, as seen *in vitro* (Bähr and Przyrembel, 1995; David et al., 1995; Shen et al., 1998), and axon growth into intact Wld^{s} peripheral nerves in certain cases is limited, possibly due to the still-intact myelin that coats these nerves (Bisby and Chen, 1990; Brown et al., 1991; Chen and Bisby, 1993a). However, it remains unclear whether PNS debris is inhibitory *in vivo*. We regularly see axons regrow while debris is present, suggesting a few theories: axons are not inhibited by PNS debris *in vivo*, or axons can grow in close contact with debris, and navigate around debris particles that block their path (Kang and Lichtman, 2013), or that Schwann cells can insulate axons from any negative effects of the debris. These are not mutually exclusive theories. However, I find the third theory attractive based on movies of regrowth in *sox10*$^{-/-}$. Regrowth in *sox10*$^{-/-}$, like in wild type, initiates while there is still debris present (Chapter 3 Figure 4). However, in *sox10*$^{-/-}$ axons extend and retract frequently, eventually extending in tangled growth patterns. Are "naked" axons in *sox10*$^{-/-}$ mutants turning in circles because contact with debris is repellent without Schwann cell protection? While I don't have enough evidence to prove this theory, I have seen axons in *sox10*$^{-/-}$ mutants turn when they encounter debris (Supplemental Figure 7). It's also likely that some other unlabeled cell, molecule, or something turns the axons, perhaps even a macrophage phagocytosing the debris. It is also possible that debris has nothing to do with the aberrant turns and misguided growth in *sox10*$^{-/-}$ mutants and that our predominant conclusion, that axons require Schwann cells for directed growth, accounts for the squirrely regrowth pattern in *sox10*$^{-/-}$: axons turn frequently because they have not stabilized on a particular trajectory. However, if direction from Schwann cells tells the entire story we might expect axons to regrow equally in all directions, including

ventrally. As in absence of Schwann cells axons usually grow in a distinctive lateral direction in the in the upper half of the myotome, where there is the least debris, this suggests that there may be some degree of debris avoidance in these mutants, and that debris may have a negative impact on "naked" regrowing axons.



Supplemental Figure 7. Regrowing axons in Tg(Mnx1:GFP); *sox10*$^{-/-}$ retract or turn after encountering axonal debris. Yellow arrow: extending axon Blue arrow: retracting axon, splayed growth cone

We show that motor axons regrow through the myotome with an average rate of .22 mm/day, comparable to what's seen in other species: in rodents and rabbits post crush axons regrow at 3-4 mm/day, and after transection 2.5-3 mm/day; clinical data indicates that humans regrow at 1-2 mm/day (Brushart, 2011), while the zebrafish lateral line, a sensory nerve, regrows at a rate of 0.72 mm/ day (Villegas et al., 2012). Our timelapse movies reveal that axon regrowth *in vivo* is variable: axons sometimes grow steadily over short distances, then halt before continuing growth; sometimes axons grow in 10-20 micron spurts in just a few minutes, but will halt again before continuing growth. To our knowledge, zebrafish provide the only vertebrate system where we can visualize the regrowth of nerves for multiple hours, especially alongside other labeled cell types,

allowing for a very detailed description of exactly how axons extend *in vivo* (Chapter 3 Figure 4).

In mammalian literature distal Schwann cells form the band of Bungner, a critical regrowth structure that provides axons with a permissive, adhesive substrate for growth (Bungner, 1891; Cajal, 1928; Nguyen et al., 2002; Scherer et al., 1984; Zuo et al., 1998). We also see that the majority of distal Schwann cells remain along the original path forming the band of Bungner, and axons regrow along the band of Bungner with great fidelity (Chapter 3 Figure 1). To determine the necessity of Schwann cells in axon regeneration we transected nerves in a series of mutants in which the Schwann cell population is genetically ablated. All mutants showed the same regenerative phenotype whereby axons regrew exuberantly, and as quickly as axons in wild type animals, but without Schwann cells axons chose aberrant trajectories. We also showed through time lapse movies that Schwann cells play a critical role early in regeneration, guiding regrowing axons to the appropriate ventral path, long before regrowing axons encounter the fixed Schwann cells that form the band of Bungner. This is discussed in detail in Chapter 3.

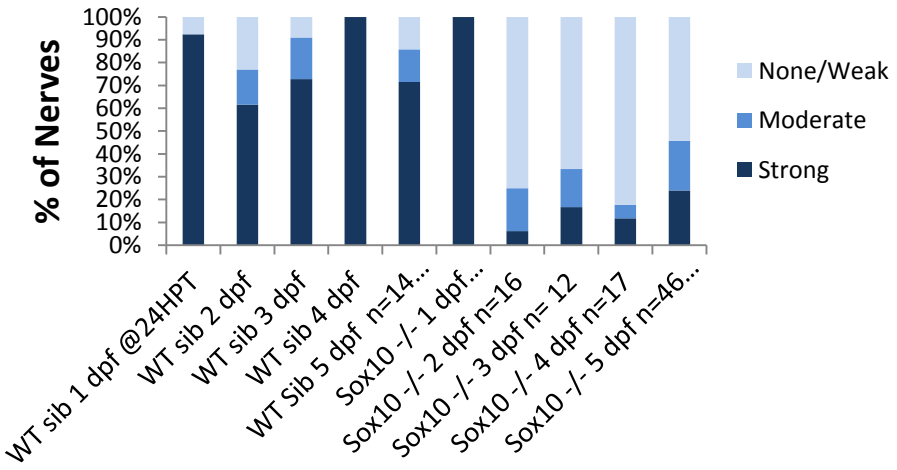
To determine whether Schwann cells guide regenerating axons in the transection gap before axons reach the band of Bungner we provided regenerating axons in the *sox10*$^{-/-}$ mutant with a permissive adhesive substrate positioned distal to the transection gap: a substitute band of Bungner, incapable of producing any Schwann cell-derived signals, and tested the ability of axons to pathfind to this substrate (details in Chapter 3). Axons did not locate the scaffold, supporting our theory that Schwann cells provide axons with directional guidance as axons regrow, before axons utilize the band of

Bungner (Chapter 3 Figure 5). This experiment underscores the uniqueness of the Schwann cell in coordinating the regenerative response. It was recently shown that Schwann cells partner with fibroblasts to form a bridge through the transection site (Parrinello et al., 2010). Our genetic ablations only affect neural crest cells, leaving fibroblasts intact. This indicates that fibroblasts cannot form a bridge without Schwann cells, and that no other cell is capable of substituting for Schwann cells in guiding axons across the transection gap: this role cannot be filled by macrophages, fibroblasts, or even signals from the end organ (Brushart, 1993; Politis et al., 1982).

So how do Schwann cells guide axons across the transection gap and to the band of Bungner? Schwann cells could produce guidance signals to lure axons across the transection gap, or form a bridge across the transection gap that connects with the band of Bungner (Cajal, 1928; Chen et al., 2005; Hall, 1986a, b; Ide et al., 1983; McDonald et al., 2006) (Parrinello et al., 2010). To test the possibility that axons only require a permissive structural guide that bridges the transection gap we provided regrowing axons with a full length, uninterrupted axon tract that extends from the spinal cord to the bottom of the ventral myotome by hemisecting nerves in *sox10*$^{-/-}$ larvae. Still, many regrowing axons extended along aberrant trajectories, confirming that axons can regrow over permissive substrates, but that axons must actively select the appropriate path back towards the band of Bungner (Chapter 3 Figure 5). While Schwann cells may form a bridge across the transection gap to the band of Bungner, our data suggest that Schwann cells also produce signals that direct regenerating axons to appropriate trajectories. Previous studies have shown that regenerating axons are tropically inclined towards the distal stump, and have shown that Schwann cells can secrete directional cues to the proximal stump (across a .5

cm gap in rats with a silastic implant (Politis et al., 1982)). Schwann cells can produce secreted neurotrophic factors, some of which have been shown to influence the direction of axonal outgrowth, such as GDNF and NGF (Arthur-Farraj et al., 2012; Cajal, 1928; Jessen and Mirsky, 2008; Schuster et al., 2010). Future studies will identify which of the many molecules that Schwann cells can produce are instrumental in guiding axons through this regenerative step.

During development axons utilize guidance cues embedded in their environment to pathfind (Tessier-Lavigne and Goodman, 1996). When does this developmental mode end, signaled by either a change in sensitivity of axons to these cues or an environment that no longer presents these cues, such that axons must rely on Schwann cells for guidance? To address this we looked at nerve regeneration in *sox10*$^{-/-}$ larvae starting at 1 dpf through 5 dpf. Nerves transected on days 2-5 resulted in misguided axons, indicating that axons have switched to requiring Schwann cells to provide guidance. At 1 dpf (24-28 hpf), axons have grown through about ¾ of the myotome, and nerves were transected in these still-extending axons at the same distance from the spinal cord as with all previous experiments. However, regeneration was so successful in nerves transected at 1 dpf that at 24 hpt transected nerves were indistinguishable from uncut nerves (Supplemental Figure 8). This data suggests that the switch from the PNS development mode to PNS adult mode starts at 2 dpf, likely when the myotome has been fully traversed. This suggests that either guidance cues embedded in the environment persist until 2 dpf, or that axons express appropriate receptors for these cues until 2 dpf.



Supplemental Figure 8. Quantification of the extent of regrowth in *sox10*$^{-/-}$ mutants from 1 dpf - 5 dpf.

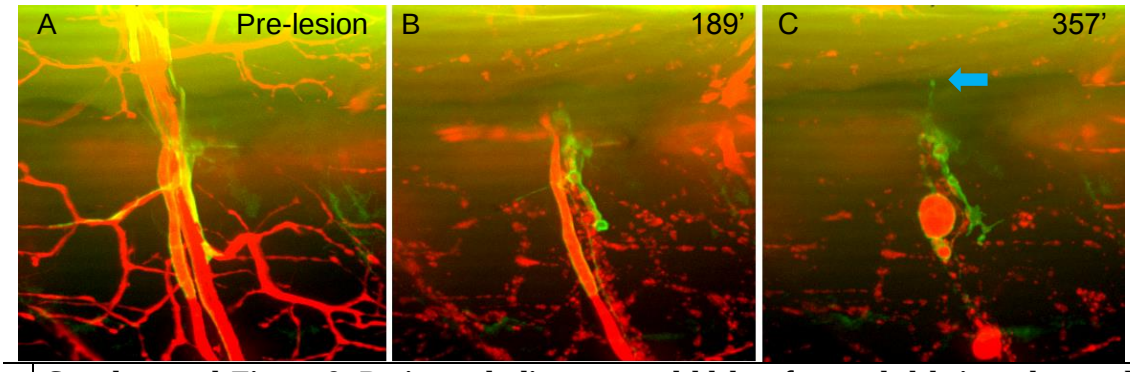
To determine whether myelination or Schwann cell maturity have a role in axon regeneration we looked at the *gpr126*$^{-/-}$ mutant. GPR126 is a G protein-coupled receptor required autonomously in Schwann cells for myelination, and in *gpr126*$^{-/-}$ mutants Schwann cell development is normal, but arrested at the promyelinating stage and will not wrap more than 1.5 times around axons to form myelin sheaths (Monk et al., 2009). We assessed degeneration timing and morphology, and regeneration pattern and extent in these mutants: all parameters appeared indistinguishable from wild type siblings (data not shown). As Schwann cells are known to downregulate myelin-related genes and de-differentiate to perform as the regenerative or bungner cell, this is not a surprising result (Arthur-Farraj et al., 2012; Zochodne, 2008).

Perineural glia, which are CNS-derived glial cells that form a blood-nerve barrier around the nerve and Schwann cells, formed cellular bridges that guided axon regeneration *in vitro* (Akert et al., 1976; Kucenas et al., 2008; Schröder et al., 1993; Weis et al., 1994a). Perineurial mini-fascicles have been observed in both the proximal and distal portions of the regenerating nerve, and perineural glia have been show to cross a

transection gap, but whether these cells are leaders or followers, and thus if they play an *in vivo* role in axon regeneration remains unclear (Binari et al., 2013; Geuna et al., 2009). We utilized the Tg(Nkx2.2a-GFP) line to label perineural glia to determine their role in axon degeneration and regeneration (Kucenas et al., 2008). At 5 dpf the perineural sheath does not appear to be fully extended and wrapped around the nerve. As nerves fragmented distal perineural glia assumed a rounded morphology, much like Schwann cells, and retracted their membranes. Also, proximal and distal perineural glia extended membranes towards the lesion site. These preliminary data suggest that perineural glia may act in some regard like Schwann cells during axon degeneration and regeneration, and may indeed produce extensions that cross or minimize the size of the transection gap (Supplemental Figure 9). Future studies will determine whether perineural glia play a role in promoting axon regeneration.

Successful regeneration requires axons to restore function to their target cells. To address whether transected motor axons restored function to fast twitch muscle fibers we utilized a well-established and quantifiable behavioral assay, the startle response, which critically depends on the simultaneous and unilateral activation of trunk muscle by spinal motor nerves, and demonstrated that regrowing axons restored functionality to their muscle targets by 48 hpt (discussed fully in Chapter 2, and Chapter 2 Figure H-O) (Burgess and Granato, 2007; Eaton and Hackett, 1984; Kimmel et al., 1974; Liu and Westerfield, 1988; Liu and Fetcho, 1999). However, it is evident from our images that there are more axons in the pre-lesion nerve than in the regenerated nerve. This indicates that a fraction of the initial innervation is sufficient to restore large behaviors like trunk

muscle contraction utilized in a swim. This may not be the case for fine motor movements such as in facial muscles.



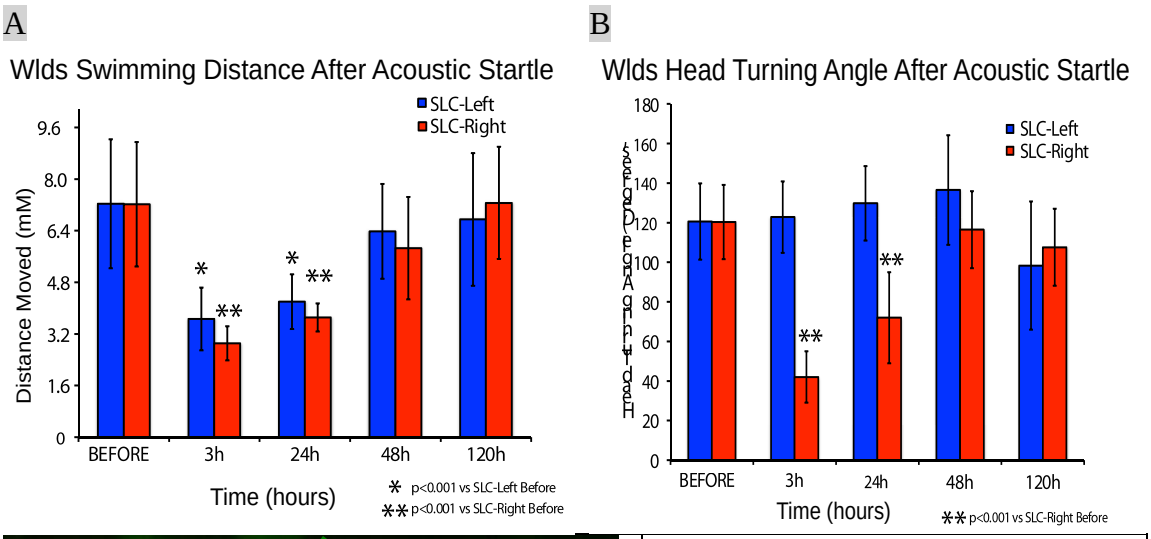
Supplemental Figure 9. Perineural glia surround blebs of axonal debris and extend processes into the transection gap following injury (arrow) in Tg(Nkx2.2a:GFP; NBT:dsRed).

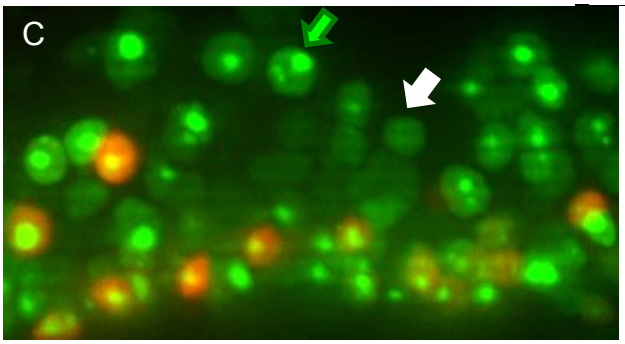
In some models Wld^{s} nerves do not regenerate well, but regeneration appears to proceed normally in Wld^{s} positive mouse sciatic nerve (Brown et al., 1991; Fruttiger et al., 1995; Lunn et al., 1989a). In our Wld^{s} line multiple axons sprout, cross the transection gap, and grow ventrally. Where axons regrow in relation to the original distal nerve is difficult to determine, as the distal, disconnected axons remain: regrowing axons appear to either utilize the remaining distal nerve as a substrate or grow very close to it (Chapter 2 Figure 3, Chapter 3 Figure 5 E-H). Our Wld^{s} line is mosaic: while most motor neurons express Wld^{s}-GFP, a subset of axons do not (Supplemental Figure 10 C). By injecting mnx1:dsred at the one cell stage, which stochastically labels motor neurons and their axons we attempted to determine whether the wild type or Wld^{s} axons are capable or incapable of regrowth in this system. We assessed the regeneration of these single axons using the following three categories: no regeneration; 10-50 um growth into the myotome; and regrowth through ¾ of the myotome (>~50 um) that recapitulates the axon's former pattern. This data is summarized in Supplemental Table 2.

Regrowth Extent of singly labeled axons	Wld^s animal, WT axon	Wld^s animal, Wld^s axon	WT animal, WT axon	WT animal, *single* WT fascicle cut
>50 um, original patterning	0	3	5	8
10-50 um	2	6	1	5
No regrowth	1	1	4	5
Total nerves cut	3	10	10	18 fascicles, not nerves

Supplemental Table 2. Regrowth extent of singly labeled axons in wild type and Wld^s mutant larvae after nerve transection, or after single fascicle transection **(last column).**

These experiments were not fruitful; we determined that individual axons are unpredictable in their regrowth, whereas regrowth was easy to parse on a population scale. We also cut single fascicles in wild type animals hoping to mimic the environment of Wld^s. Regrowing axons in Wld^s will encounter intact axons on their original path; by cutting a single fascicle that included our singly labeled axon, and leaving the rest of the nerve intact, we hoped to see how these intact axons on the original path affected the regrowth of the singly labeled axon. This too wasn't very telling. We also performed the aforementioned behavioral assay in our Wld^s line: by 48 hpt all parameters had reached pre-lesion levels, suggesting that regrowing axons restored functionality to their muscle targets even when these targets remained innervated by the previous, disconnected distal motor nerve (Supplemental Figure 10A, B). This also suggests that the exact location of neuromuscular synapses is less critical in an en passant system, as these muscles are doubly innervated by old, non-functional synapses, as well as new, functional synapses from the regenerated axons.

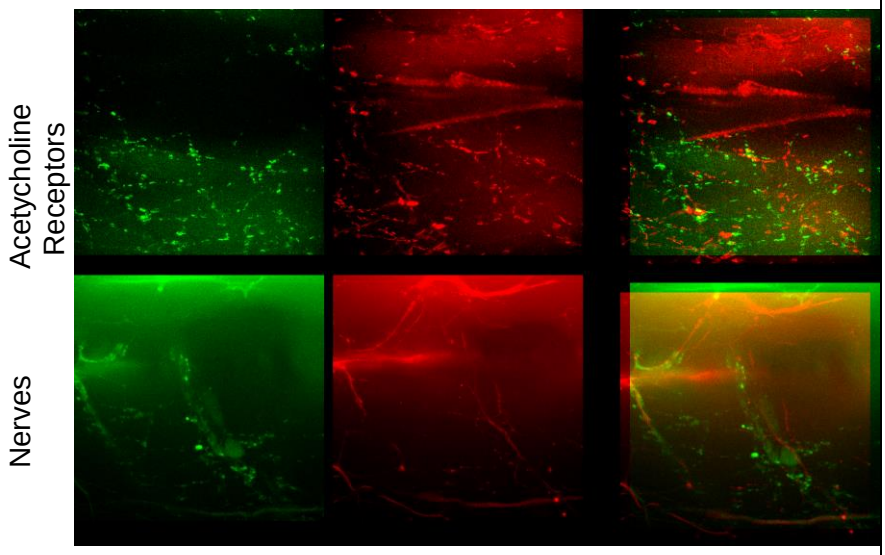




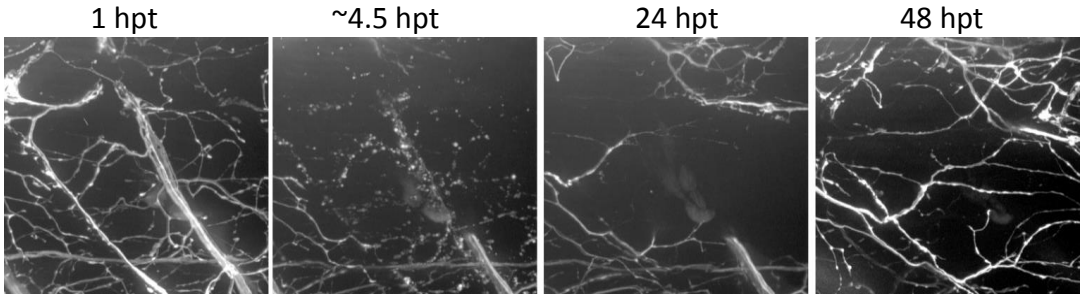
Supplemental Figure 10. Axon regeneration in Wld^{s}. (A,B) Startle parameters in Wld^{s}. (C) Wld^{s} transgenics are mosaic, green arrow: Wld^{s}+ motor neuron; white arrow: wild type motor neuron. Red neurons are stochastically labeled with mnx1:dsRed in Tg(Mnx1:GFP; Mnx1:Wld^{s}-GFP)

To determine where regenerating axons formed synapses in relation to their original synaptic sites we visualized acetylcholine clusters (AChRs) pre and post lesion, which are concentrated at neuromuscular junctions (Sanes and Lichtman, 2001). Utilizing the Tg(AchR-YFP; Nbt-dsRed) line that labeled these clusters *in vivo*, we found that AChR clusters disintegrate post lesion, and reformed where axons regrew. However, the new locations do not overlap with the original locations (Supplemental Figure 11). This suggests that either these synapses will be pruned later, as seen in other systems (Salzer, 1999), or, as regeneration in the Wld^{s} mutant suggests, precise neuromuscular synapse location isn't as critical en passant systems as long as communication is restored to the target cell, which also prevents degeneration of the tissue (Eisen et al., 1986; Myers et al.,

1986; Westerfield et al., 1986b; Zochodne, 2008). Interestingly, we also see a lot of compensatory sprouting in our system. I have not quantified whether there is an increase in compensatory sprouting in *sox10*$^{-/-}$ mutants compared to wild type. Compensatory sprout is seen frequently, though not always (Supplemental Figure 12).



Supplemental Figure 11. After regeneration acetylcholine receptors (AChRs) do not cluster in the same location as pre-lesion sites. Tg(AchR-YFP;Nbt-dsRed) post and 24 hpt images were aligned by axons and AChR clusters from the adjoining segment on the bottom left.



Supplemental Figure 12. Adjacent hemisegments can send compensatory sprouts into hemisegments with nerve damage. Tg(Mnx1:GFP); *sox10*$^{-/-}$

During development motor axons pathfind using a multitude of guidance cues, among them Netrin and its receptor DCC, expressed on axon growth cones (Hale et al., 2011; Lim et al., 2011) Previous groups have found that Netrin is expressed by band of

Bungner Schwann cells, and that regenerating motor axons, and Schwann cells, both express DCC (Madison et al., 2000; Webber et al., 2011). We investigated whether DCC has a role in guiding regenerating motor axons *in vivo*. We found that DCC mRNA is present in zebrafish motor neurons, and analyzed axon regeneration in a *dcc*$^{-/-}$ mutant. While the extent of axon regeneration appeared normal (axons regrew through the entire length of the ventral myotome in 90% of siblings and 78% of *dcc*$^{-/-}$ mutants, Chapter 3 Figure 6), we observed that many axons in *dcc*$^{-/-}$ chose an aberrant lateral path. We conclude that DCC is one of many guidance molecules that have a role in axon pathfinding during regeneration.

Interestingly, work by the Zochodne group suggests that it is not axons, but Schwann cells that require DCC for axon regeneration (Webber et al., 2011). While their work does not prove this point, it is interesting to consider the hypothesis that the Schwann cell, which extends from the proximal stump alongside or ahead of axons (Chen et al., 2005; Hall, 1986a; Son et al., 1996; Torigoe et al., 1996) could be responsible for interpreting guidance cues instead of axons (Webber et al., 2011). During development Schwann cell precursors and axons both utilize the same environmental cues to pathfind, and ablating either cell type does not affect the other cell type's ability to pathfind through the myotome (Banerjee et al., 2011)(Chapter 2 Figure 7). This indicates that both cell types express receptors that interpret guidance cues, and suggests that it is possible for both cell types to re-express these receptors during regeneration. As both cell types leave the proximal nerve stump together, two future experiments will elucidate which cell type definitively leads across the transection gap. First, detailed live imaging with membrane tagged axons and Schwann cells will show which cells lead and follow.

Second, in mutants lacking important regenerative guidance receptors, such as DCC, cell type specific rescue in Schwann cells or axons will determine which cell type must express the receptor to rescue the directionality of regrowth. If it is Schwann cells and not axons that lead the way, and Schwann cells also express DCC and other guidance cue receptors, the next mystery will be to determine how Schwann cells convey directional information to regrowing axons.

CONCLUDING REMARKS

In summary, this work establishes an *in vivo* system in larval zebrafish for studying motor nerve degeneration and regeneration. We show that as in mammals, myelinated zebrafish peripheral motor nerves undergo Wallerian degeneration, followed by functional regeneration, and that the genetics of motor axons fragmentation is conserved from zebrafish to mammals. We provide the first minute-by-minute account of the destruction speed and synchrony of individual motor axons, macrophage recruitment, and changes in Schwann cell morphology in a live intact vertebrate animal. We uncover a previously uncharacterized plasticity of macrophage behavior as they interact with injured nerves. We investigated how regenerating axons cross an injury-induced transection gap and select appropriate trajectories. Using zebrafish with genetically ablated Schwann cells and through *in vivo* time lapse movies we find that Schwann cells are critical for guiding axons to appropriate trajectories at the outset of axon regrowth. We show that trophic support from Schwann cells is dispensable, as axons extend to great lengths in the absence of Schwann cells. By providing an axonal scaffold and genetically

ablating Schwann cells we find that a physical adhesive substrate is insufficient to guide regenerating axons, and that Schwann cells produce signals that guide axons to the appropriate trajectory. Lastly, we examined mutants lacking the netrin receptor DCC and saw that regenerating axons were misguided.

In conclusion, our results demonstrate that Schwann cells are key coordinators of axon guidance during regeneration. Schwann cells not only form the band of Bungner, but also produce signals early during regeneration that guide newly sprouted axons to the appropriate path. These signals could be secreted from Schwann cells to entice axons to appropriate trajectories, or if Schwann cells bridge the gap before axons, then these cues could be presented on the surface of Schwann cells for axons to recognize as they utilize a Schwann cell tissue bridge over the transection gap. As tissue that has been denervated for too long cannot be successfully reinnervated, is it critical that axon regeneration proceed with as much speed as can be mustered. Thus, guiding axons quickly and efficiently to the appropriate path is a critical step in successful regeneration. Further studies will elucidate additional molecular cues in this process and determine which of these cell types must express receptors for these cues.

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